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PATHS OF RENAL INFECTION.

C. H. KELLAWAY, C. J. O. BROWN AND F. ELEANOR WILLIAMS.

From the Walter and Eliza Hall Institute, Melbourne.

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INFECTING organisms may conceivably reach the kidneys by the blood-stream, by the lymphatics or by direct extension along the wall or through the lumen of the ureter. The work of Wysokowitsch (1886), Sherrington (1893) and many others clearly attests the possibility of infection by the blood-stream, and in this connection Brewer (1911) and Lepper (1921) have emphasized the importance of injury to the kidney.

The connection between the lymphatics of the kidney and bladder through those of the ureter has been demonstrated by Sakata (1903), but much of the evidence in favour of spread along these channels is inadequately controlled in regard to possible blood infection. Stewart (1910) and Sweet and Stewart (1914), for example, in experiments with dogs, found that if the ureter were implanted into the bowel, the course of the infection which resulted could be traced in peri-ureteral areas of necrosis and purulent infiltration. When they avoided obstruction at the point of entry of the ureter by passing it through the pancreatic duct the corresponding kidney was not infected, but in their positive experiments obstruction may have determined localization from the blood. The experiments of Eisendrath and Schultz (1916), which favour ascending infection by the lumen of the ureter in cases of obstruction and by the lymphatics in simple infection of the bladder, were only controlled by blood-cultures taken at autopsy. Walker (1922) and Thiele and Embleton (1914) appear to have demonstrated ascent through the lymphatics in the guinea-pig. A loopful of *B. prodigiosus* was placed within the lumen of the urethra, and after 10 to 16 hours the organisms could be recovered from the tissues at the upper end of the ureter, but not from the blood or urine. It is worthy of note that in no case where blood infection has been excluded have the renal lesions, supposedly produced by lymphatic spread, been associated with the presence of organisms within the pelvis of the kidney.

The evidence for ascending infection by transportation in the column of urine occupying the lumen of the ureter is more uncertain. Such ascent is normally opposed by the downward flow of urine and by the arrangement of the musculature round the ureter when it enters the bladder, though Draper

and Braasch (1913) failed to cause infection of the kidney in dogs after slitting the ureteric orifice. The work of earlier experimenters was not adequately controlled by blood-cultures, though there are some observations which cannot be dismissed from consideration on these grounds. Guyon and Albarran (1890) injected charcoal into the bladders of dogs and rabbits, and with complete retention found particles of charcoal in the pelvis after 48 hours, though only in minute quantities. They held that since 48 hours was necessary for the ascent, anti-peristalsis was not likely to explain the phenomenon, but that light particles might float from the bladder into the ureter and be carried by eddies or to-and-fro currents in the very slowly descending stream of urine. Lewin (1898) and Lewin and Goldschmidt (1893) injected milk and coloured fluids into the bladders of rabbits, and claimed to have observed regurgitation from the bladder into the pelvis of the kidney when the urethra was obstructed.

The work of two more recent observers whose experiments favour ascent along the lumen is also not wholly free from objection on the ground of possible blood infection. Brewer (1911) injected organisms into the bladders of dogs and produced artificial retention for 24 hours, which was then relieved. The animals, killed after various intervals, showed gross pyonephrosis. David (1918), who also used dogs for his experiments, found that when coliform organisms were introduced into the bladder after ligation and division of one ureter, the pelvis of the kidney whose ureter was unobstructed became infected, while the hydronephrotic kidney and the blood-cultures taken at autopsy were sterile. In animals in which in addition the urethra had been partially obstructed, and which were killed 1 to 5 days after the operation and infection of the bladder, the urine in the pelvis of the unobserved kidney was infected in ten out of eleven experiments. As will be shown in the present paper, neither negative blood-cultures at autopsy nor a sterile hydronephrosis can be relied upon to exclude blood infection. According to David, peri-ureteral infiltration is not synonymous with infection of the urinary stream, and his experiments are distinctly unfavourable to lymphatic spread as the explanation of his findings.

In the present paper we bring forward evidence in support of the possibility of the occurrence of ascent along the lumen of the ureter as the cause of pyelitis when the urethra is obstructed or the nervous control of the bladder is disorganized.

METHODS.

All operations were carried out with strict aseptic precautions under ether anæsthesia. Our experiments with infecting organisms were performed on female guinea-pigs of 500 to 600 gm. weight, and to obtain organisms pathogenic for this animal we injected 1 c.c. of a broth emulsion of fresh guinea-pig fæces into the bladder of a guinea-pig in which the left ureter was ligated and the urethra partially obstructed. At autopsy 48 hours later there was a right-sided pyelonephritis with lines of purulent infiltration running through the kidney to the capsule. From the bladder and from the pelvis of the right kidney a Gram-negative bacillus, a staphylococcus and a hæmolytic streptococcus were isolated. The first-mentioned organism was non-motile, fermented neither glucose nor lactose, gave acid but no gas in mannite and caused no hæmolysis on blood-agar. A diphtheroid and a *Staphylococcus aureus* were also obtained from accidental infections occurring in the course of our work. The organisms in a saline emulsion were introduced into the bladder by catheter after cleaning up the orifice of the urethra with antiseptics. Sterile glycerine was used as a lubricant.

To cause partial obstruction we adapted the device used by Bolton (1910) for obstruction of the inferior vena cava. We exposed the urethra above the symphysis pubis and passed round it a short piece of split rubber catheter, which had an internal diameter of 3·5 mm. when its ends were in contact. In this way a sufficient grade of obstruction was produced to cause distension of the bladder, though the obstruction could be overcome by pressure on the abdominal wall. Disorganization of the nervous mechanism of micturition was produced by destroying or excising the nerve-roots in the spinal canal below the level of the iliac crests. A uniform functional impairment of the bladder resulted, which was unaccompanied by any paralysis of the hind limbs. The bladder became distended, but slight pressure on the abdominal wall or contraction of the abdominal muscles caused forcible expulsion of urine. One ureter was ligated in all these experiments, either close to the bladder or through the flank. In some cases single ligation, in others double ligation and division was practised.

Autopsies were carried out aseptically. Cultures were taken from the heart and bladder, of the cortex of the kidneys after cauterizing the surface, and of the urine obtained from the pelves of the kidneys (after tying off the unligated ureter) by puncture, either directly or through the kidney after deeply cauterizing it. The culture media used throughout were "hormone" broth and "hormone" agar. Blood-cultures were kept for a week before finally being discarded.

To ascertain whether when the sacral nerve-roots are destroyed or the urethra obstructed, lymphatic ascent can be demonstrated, we carried out a few experiments on the general plan devised by Thiele and Embleton, recognizing that the time relations might be wholly different in our experiments. In these we controlled our results by taking hourly blood-cultures, 0·2 to 0·5 c.c. of blood obtained from partial division of an ear vein being added to 5 c.c. of broth. Contaminations were very infrequent. In series of animals killed at different periods we avoided taking more than 6 blood-cultures from any one animal, by planning them so as to cover at hourly intervals the whole time over which the group of experiments were spread. As a further control, cultures of the heart blood, lung, spleen, liver, cortex of the kidney and gall-bladder were made at autopsy. The wounds, which had been painted with iodine and covered with collodium, were cultured, and were usually negative in the absence of blood-infection. The urine in the pelvis of both kidneys, the kidney capsules, the upper and lower halves of the ureters and the pre-aortic, lumbar and hypogastric glands were also cultured.

Another group of experiments was carried out with suspensions of easily recognizable solid particles or of pigments insoluble in water. The animals investigated were the guinea-pig, rabbit and cat. After the sacral nerve-roots had been destroyed, or before the urethra was obstructed and while the animal was still under the anæsthetic, these sterile suspensions were injected into the bladder. The animals were killed at varying periods, and their kidneys were removed after double ligation of the ureters and division between the ligatures. The urine was withdrawn from the pelvis with a Pasteur pipette, and the kidney was subsequently laid open and the mucosa lightly scraped. The pelvic urine and the scrapings were examined for the presence of the substance which had been introduced into the bladder. In some experiments we fixed the ureter, dehydrated, cleared and mounted it, and in other cases serial sections of the ureter and pelvis were cut and examined.

EXPERIMENTAL.

In the guinea-pig, as in other animals, the bladder with uninjured mucosa and intact nervous connections exhibits a high degree of resistance to the action of pathogenic micro-organisms injected into its lumen.

A catheter specimen for culture was first obtained and an emulsion of streptococci, diphtheroids or staphylococci was injected into the bladder, care being taken to avoid damage to the mucous membrane of the urethra. In a few experiments a slight cystitis was produced. Catheter specimens for culture were taken on alternate days. In some cases the urine was again sterile by the fourth day, but in others the organisms (diphtheroids and staphylococci) persisted in the bladder or urethra for as long a period as a month, without causing pyelitis or cystitis. These animals remained in good condition—pus and blood were absent from the urine, and the kidneys were normal in appearance and sterile at autopsy.

When the introduction of organisms into the bladder is accompanied by obstruction to the outflow of urine through the urethra or by injury to the nervous connections of the bladder, cystitis is invariably caused. Whether or

no there is pyelitis in addition appears to depend on the nature of the infecting organism.

(a) *Infection of the Bladder Associated with Partial Obstruction of the Urethra.*

Partial obstruction does not by itself lead to infection in the urinary tract. In five experiments in which obstructions were produced without simultaneously infecting the bladder, the urinary tract was sterile by culture when the animals were killed 48 to 72 hours later.

In the subjoined table are the results of a series of 14 experiments in animals examined 2 to 4 days after partial obstruction of the urethra and ligation of the right ureter. In Nos. 1, 2 and 3 *Staphylococcus albus*, in 4, 5, 6, 7 and 8 streptococci, in 9, 10, 11, 12 and 13 diphtheroids, and in 14 and 15 Gram-negative bacilli were introduced into the bladder. No. 7 recovered from the infection, but died 20 days later with acute retention, caused by the impaction of a stone in the urethra at the site of the stricture.

No. of experiment.	Cultures from pelvis of left kidney.	Cultures from bladder.	Remarks.
1	*Staphylococcus albus* and Gram-negative bacillus		Killed after 48 hours. *S. albus* isolated from blood. Right kidney pelvis sterile.
2	*S. albus*, diphtheroids, Gram-negative bacilli and streptococci		Killed after 4 days. Ureter tied 5 days before urethral obstruction and introduction of organisms. Blood and right pelvis sterile.
3	Sterile	*S. aureus*	Killed after 2 days. Stitch infection in wound. Ureter tied 8 days before. *S. aureus* from wound and right pelvis.
4 and 5	Streptococci		Killed after 2 days. Ureter tied 21 days before. Pelvis of right kidney sterile. Streptococci isolated from blood.
6	Streptococci and coliform bacilli		Died after 4 days. Ureter tied 15 days. Heart-blood and right pelvis sterile.
8	Sterile	Streptococci	Killed after 2 days. Blood and right pelvis sterile.
9	Diphtheroids and streptococci		Died after 36 hours. Blood, cortex of both kidneys and urine from right pelvis sterile.
10	Diphtheroids	Diphtheroids and streptococci	Died after 2 days. Blood sterile. Urine from right pelvis contains diphtheroids.
11	Diphtheroids. Large Gram-positive cocci		Died after 2 days. Ureter tied 11 days before urethra obstructed. Right pelvis sterile. Blood-culture—diphtheroids.
12	—	—	Died after 2 days. No cultures: membranous pyelitis and ureteritis on untied side.
13	Diphtheroids	Diphtheroids and *S. albus*	Killed after 3 days. Diphtheroids in blood and right pelvis, and *S. albus* in right pelvis in addition.
14	Gram-negative bacillus		Killed after 3 days. Blood and right pelvis sterile.
15	—	—	Killed after 4 days. All cultures sterile.

In seven experiments, Nos. 1, 3, 4, 5, 10, 11 and 13, blood infection was present; in five of these this was indicated by the blood-culture and in three by the infection of the tied-off kidney. Neither negative blood-cultures at autopsy, nor the sterility of the kidney, from which the outflow has been obstructed by ligation of the ureter, can therefore be relied upon to exclude blood infection. In Nos. 4, 5 and 11, in which positive blood-cultures were obtained, the kidneys had been tied off from 11 to 21 days, and the greatly diminished blood-supply to these hydronephrotic kidneys may have accounted for their failure to become infected.

We investigated this point further, and found that when one ureter was tied with or without urethral obstructions and organisms were injected intravenously, the obstructed kidney was not invariably infected.

In four experiments we injected 250 million diphtheroids intravenously 24 and 48 hours after partial obstruction of the urethra and ligation of the right ureter, and 2 and 3 days respectively after simple ligation of the right ureter. In two experiments with streptococci the intravenous injection was made 24 hours after ligation of the right ureter alone and after ligation of the ureter with urethral obstruction, and in three experiments with *Staphylococcus albus* the injection was made in 2, 1 and 4 days after ligation of the right ureter, and in one, a day after ligation of the ureter and partial obstruction of the urethra. The animals were killed in from 1 to 4 days after the injection. The hydronephrotic kidney was infected in 4 out of the 5 experiments in which the ureter had only been tied 24 hours. When the injection was made 2, 3 or 4 days after the operation the kidney did not become infected. These experiments further emphasize the fact that blood-cultures taken at autopsy cannot be relied upon to detect bacteriæmia during life, as in only 2 cases were organisms recovered from the blood.

In experiments Nos. 1, 2, 6, 9 and 11 in the table, where the bacteria in the bladder are of more than one kind, exactly the same flora was found in the pelvis of the kidney on the unligated side. In 10 and 13 a mixed culture was obtained from the bladder, but only one organism was isolated from the pelvis of the untied kidney. The coincidence of the flora of the bladder and unobstructed pelvis may be explained by successive or simultaneous blood infections, by lymphatic spread, or more simply by ascent along the lumen of the unobstructed ureter. If this latter explanation be correct, the exceptions, which are easily explained by the theory of blood-borne infection, must be regarded as cases in which the secondary infection of the bladder has been acquired too late to give time for ascent, or accounted for alternatively by assuming that both organisms have reached the pelvis, but that only one has found the new conditions suitable for continued growth.

A few experiments in transplantation of the ureter were made in order to provide as a control an unobstructed kidney which was not in communication with the infected bladder. The operation was performed by excising a portion of the trigone of the bladder, and fixing it in its new site by a single suture passed through the bladder mucosa adjacent to the ureteral papilla. The bladder was repaired with a double layer of sutures. As sites of implantation the abdominal wall and the vagina were tried, but it was found to be impracticable to avoid some degree of obstruction of the transplanted ureter. After a few days, when the wounds had healed, the bladder was infected and the urethra partially obstructed. The result of one such experiment is recorded:

PROTOCOL 1.—*Guinea-pig (female).* 550 *gm.*

19.6.25. Left ureter implanted on the abdominal wall.
28.6.25. Slight phosphate deposit; some irritation of the skin round the ureteral orifice.
29.6.25. *Staphylococcus albus* injected into the bladder; urethra partially obstructed.
8.7.25. Animal in poor condition and somewhat wasted; killed. Left ureter and pelvis slightly dilated and contained blood-stained urine; right ureter not dilated neither kidney grossly abnormal; bladder wound well healed. From the pelvis of the right kidney *Staphylococcus albus* was recovered in pure culture; from the pelvis of the left kidney diphtheroids and enterococci, and from the bladder urine staphylococci and enterococci were grown. The heart-blood was sterile.

While the findings in this experiment may be readily explained by supposing three separate blood infections, it seems more likely that ascending infection, either by the lymphatics or the lumen, is the explanation of the difference in the bacterial flora on the two sides.

(b) *Infection of the Bladder after Excision of the Sacral Nerve Roots.*

In 9 animals we destroyed the sacral nerve roots, ligated the right ureter and injected organisms into the bladder. Of 6 in which diphtheroids were used, in 4 the urine from the left pelvis contained the organism when the animals were examined 24 to 72 hours later. Though streptococci were found with diphtheroids in the bladder in three cases, in only one were they present in the unobstructed pelvis. The remaining two experiments with diphtheroids and three others in which streptococci and staphylococci were used were negative. In all cases cultures from the blood and the urine in the right pelvis were sterile. The results in these, as in the experiments in which the urethra was obstructed, may equally well be explained by blood or lymphatic infection or by spread along the lumen of the ureter, and afford no clear indication as to which course the organisms pursued.

The observations of a number of workers who have injected bacteria into the blood-stream of normal animals indicate that several hours elapse before the organisms appear in the urine. We injected an emulsion of diphtheroids into the jugular veins of guinea-pigs in which the urethra had been partially obstructed or in which the sacral nerve roots had been destroyed, and found that at least 6 hours were required before diphtheroids could be grown from the bladder urine.

Six guinea-pigs in which the urethra was partially obstructed received 0·25 c.c. of a thick emulsion of diphtheroids intravenously and were killed 3, 4, 5 and 6 hours after the injection. In all cases diphtheroids were recovered from the heart-blood at autopsy and, in all but the two animals killed 6 hours after the injection, the urine in the bladder was sterile. Another group of eight animals in which the sacral nerve roots were destroyed were similarly injected and killed 2, 3, 4, 5, 6 and 7 hours after the injection. In all these the blood-cultures at autopsy were positive, though in none of them were the organisms recovered from the urine.

When organisms are introduced into the bladder, the pelvis of the unobstructed kidney becomes affected too early for this result to be explained by blood infection.

In ten guinea-pigs, in which the sacral nerve roots were destroyed, the right ureter ligated through the flank and the bladder infected with diphtheroids, the urine in the pelvis of the left kidney was found to contain diphtheroids in one animal killed 2 hours, in one 3 hours, in

two 4 hours, in two 5 hours and in one 6 hours after injection. In all of these the urine from the pelvis of the right kidney and the heart-blood was sterile, and diphtheroids were recovered from the bladder and from the right ureter below the ligature. The three remaining animals were killed, two immediately and one 1 hour after the injection. In these the urine from both kidneys and the heart-blood was sterile, diphtheroids being recovered from the bladder only.

Another group of ten animals, in which the urethra was partially obstructed and in eight of which the right ureter was ligated, gave similar results. Of four killed after 4 hours, in three diphtheroids were recovered from the urine in the pelvis of the left kidney. In two killed after 3 hours and in one killed after 2 hours the urine of the left pelvis contained diphtheroids; while of three killed after 1 hour, in two the left pelvis was sterile. The urine from the right kidney was sterile in all cases in which the corresponding ureter was tied, and in one animal only had ascent on the unobstructed side failed to occur after the second hour.

Under the conditions of our experiments, localization in the pelvis of the unobstructed kidney occurs regularly within 2 hours. The possibility that during the manipulations involved in finally chloroforming the animals bacteria might be forced by pressure up to the pelvis, or that the force of the injection might cause ascent up the lumen of the ureter, is excluded by the negative findings in animals examined immediately and 1 hour after the injection. Ascent after death is excluded by our unvarying practice of tying the ureters at autopsy before puncturing the pelvis, and further by our inability in nearly all cases to cause dyes to flow out from the cut ureters by putting up large pressures within the bladder. The regularity with which diphtheroids ascend when contrasted with the irregularity of ascent by other organisms, as seen in the longer experiments reported earlier in this paper, suggests that in the guinea-pig diphtheroids more readily establish themselves in the pelvis.

(c) *Experiments on Lymphatic Spread.*

We attempted in two experiments to reproduce Walker's uniformly positive results in normal guinea-pigs in which *B. prodigiosus* was introduced into the urethra. In animals killed after 12 and 16 hours the bladder contained *prodigiosus*, but the remaining cultures were sterile. In nine other experiments the organisms were introduced into the bladder. In five of these the urethra was partially obstructed and the right ureter tied. The animals were killed after 4, 8, 10, 12 and 15 hours. In the remaining four the sacral nerve-roots were destroyed, the right ureter ligated, and the animals killed 6, 12, 18 and 24 hours after injection. In the latter group the blood-cultures were all negative, and at autopsy *prodigiosus* was isolated only from the bladder, except in the animal killed at 18 hours, in which the left lower ureter likewise yielded a positive culture. Of the five animals in which the urethra was obstructed positive blood-cultures were obtained in the 8- and 10-hour (from the fifth hour onward) and in the 15-hour experiment (from the eleventh hour onward). The animal killed after 12 hours presented negative blood-cultures, and *prodigiosus* was isolated from the bladder only at autopsy. In the guinea-pig killed at the fourth hour *prodigiosus* was obtained from cultures of the capsules of both kidneys and from the ureter on the left side. As the urine from the pelvis on that side was infected the ureteral cultures must be discounted. Except in this single instance, we failed to find any evidence of the type of spread described by Walker and Thiele and Embleton.

In similar experiments with diphtheroids, which, unlike *prodigiosus*, fairly regularly infects the pelvis of the kidney on the unligated side, we had to rely upon cultures from the kidney-capsules and from the lymph-glands to provide evidence of lymphatic spread. In guinea-pigs, in which the only operative procedure was ligation of the right ureter, examined at 8, 12 and 21 hours after injection of organisms into the bladder, all the cultures at autopsy except those from the bladder were sterile. In one animal, killed after 14 hours, streptococci were found in the blood-culture taken from the ear at the thirteenth hour, and at autopsy the operation wound, the kidney-capsules and both ureters were found to be infected with this organism. As regards the spread of diphtheroids this experiment was, like the others, negative. Of seven other guinea-pigs in which the sacral nerve-roots were destroyed, the right ureter ligated and diphtheroids introduced into the bladder, one was killed 3 hours, one 4 hours, two 8 hours, one 10 hours, one 12 hours and one 21 hours later. In three, one 8-hour and the 12- and 21-hour animals, blood infection was present, and in none of them was there any evidence of lymphatic spread.

(d) *Experiments with Suspensions of Particles Insoluble in Water.*

The search for particles is much more inconclusive than the demonstration of the presence of living organisms by culture, but the occurrence of only one particle in the pelvis of the kidney in one amongst even a large number of negative experiments must be regarded as significant evidence of ascent along the lumen, if transport by the blood or by the lymphatics can be excluded.

In guinea-pigs our results with suspensions of carmine and lycopodium wholly paralleled our experience with diphtheroids. In five experiments in which the urethra was partially obstructed and a suspension of carmine introduced into the bladder, particles of carmine were invariably present in the pelvis of one or both kidneys after from 2 to 3½ hours. In two experiments with lycopodium suspended in saline with the aid of a trace of bile-salt, the particles were present in the pelvic urine when the animals were killed 24 hours later. When the sacral nerve-roots had been destroyed ascent of the pigment was not so regular, but occurred in four out of eight experiments in which the animals were killed from 2 to 4 hours after the injection. In these experiments particles were invariably found within the lumen of the ureter and pelvis and never in the wall of the ureter or in the accompanying lymphatics. Furthermore, carmine particles were never found in the pelvis of a kidney in which the ureter had been tied even after 24 hours.

In rabbits and cats the conditions upon which ascent depends are very different from those which obtain in guinea-pigs. The last-named animal micturates several times in the day, whereas the cat and the rabbit only do so about once a day, so that the effects of obstruction or interference with micturition are much less quickly felt in them. In three cats with obstruction of the urethra examined after 24, 40 and 50 hours, in three similar experiments with rabbits examined after 40, 43 and 46 hours, in two cats, in which the sacral nerve-roots had been destroyed, examined after 40 and 48 hours, and in a rabbit examined 48 hours after destruction of the sacral nerve-roots and injection of carmine into the bladder, no carmine particles were found in

the pelvis of either kidney. In only one experiment with carmine did we obtain a positive result. We destroyed the sacral nerve-roots in a young cat and kept the animal alive and in good condition for 6 weeks, daily expressing the urine from its bladder. We then injected carmine into the bladder and examined the animal at the end of 48 hours. A single particle of carmine was found in the pelvis of one kidney. Another cat in which we performed a similar experiment, but in which we examined the animal at the end of 24 hours, was negative.

It occurred to us that Guyon and Albarran's positive results with carbon might have been due to the relative lightness of these particles, and in three experiments, in which we injected a mixture of carmine and lampblack into the bladder, black particles were present in the pelvis on both sides, though the carmine had failed to ascend. The use of lampblack was, however, open to objection, since particles similar in appearance were occasionally to be found in our preparations in both cats and rabbits in which the substance had not been introduced into the bladder. Further, in one rabbit, in which one ureter was tied and the urethra obstructed, they were found in large numbers in the pelvis on the ligated side. We next tried a watery emulsion of olive oil containing "oil-blue" in solution. Blue particles were invariably present in the pelvic urine of both cats and rabbits with obstruction or after spinal operations as early as 20 hours after the injection into the bladder. However, the particles seen were solid masses and not oil-globules stained blue, and furthermore we were able to demonstrate that similar blue particles were constantly present in the pelvic urine of kidneys whose ureters had been tied before the injection and must have been excreted from the blood. We finally selected lycopodium suspended in saline, using a trace of bile-salt to lower the surface tension sufficiently to permit of the particles being wet by the liquid. Using this preparation we performed five experiments in rabbits in which the urethra was obstructed, which were examined 24, 44 and 48 hours after the injection. Only one of these was positive, a single particle of lycopodium being found in the 44-hour experiment.

CONCLUSIONS.

In the guinea-pig, when the urethra is partially obstructed or the nervous mechanism of micturition disorganized, the introduction of pathogenic organisms into the bladder is followed within 2 to 3 hours by the ascent of the organisms into the pelvis of the kidney. This ascent can also be demonstrated by means of carmine particles. It occurs too rapidly to be accounted for by blood infection, nor is there any evidence that it takes place by way of the lymphatics of the ureter. In rabbits and cats ascent along the lumen of the ureter is much more difficult to demonstrate and is of very infrequent occurrence.

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THE RETICULO-ENDOTHELIAL SYSTEM AND HÆMOLYSIN FORMATION.

GEO. R. ROSS, M.B., CH.B., PH.D., D.P.H.,

Rhodesian Research Fellow.

From the London School of Hygiene and Tropical Medicine.

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MUCH attention has been given in recent years to the function of those groups of endothelial cells which are found so widely distributed throughout the body and which are spoken of collectively as the reticulo-endothelial system. They have come to be regarded as playing an important part in the breaking-down of hæmoglobin and the formation of hæmosiderin and bile-pigments.

A second function has also been ascribed to them in that they are regarded by some as the seat of antibody formation.

The importance of this hypothesis is obviously apparent, both from the theoretical and practical point of view, and the evidence on which it is based demands careful scrutiny.

It is believed that the primary step towards such antibody formation lies in the absorption from the blood-stream of particulate and colloidal matter by the reticulo-endothelial system. It has been stated that the absorption of a colloidal dye, such as carmine, prevents the absorption of a similar colloidal substance, such as colloidal iron, when this is injected subsequently. Thus it would seem as if the system had only a limited capacity for the absorption of such substances, and when actively engaged in the absorption of one colloid that it is incapable of absorbing a second similar substance introduced at a later period.

Antigens can certainly be regarded as colloidal in nature in so far that they consist of particulate matter in fine suspension, and believing that such is taken

up by the reticulo-endothelial system, certain investigators have utilized the limited or selective capacity of the system in order to attempt to localize the site of antibody formation in that system.

The method of experiment has been generally first to "block" the system by saturating the blood-stream with some indifferent colloidal substance which is absorbed by the constituent cells, or actually to destroy the cells by means of some radio-active body. It has been shown that if such is injected into the body it is in part at least retained by the endothelial cells, and that this retention ultimately leads to destruction of the cells because of radiations which are given off by such retained particles.

When the "blocking" or destruction of the system is thought complete, antigens capable of the production of agglutinins, precipitins and hæmolysins have been injected, and the antibody response in the treated animals compared with that in non-treated controls.

It should be pointed out that these experiments are made much more definite in their aim than those of Walbum and Mörch (1923), who attempted to influence antibody formation by means of injections of manganese, or those of McIntosh and Kingsbury (1924), who employed salvarsan, beryllium chloride, colloidal lead and colloidal silver in a similar experiment.

Bieling (1923), however, has conducted experiments in which he attempted to saturate the reticulo-endothelial system by means of saccharate of iron. He found that preliminary treatment with this did not prevent subsequent antibody formation. Mice, however, in which splenectomy had been performed and which were similarly treated, did show an inhibition of antibody formation.

Gay and Clark (1924) "saturated" a series of rats and rabbits by daily intraperitoneal injections of trypan blue over a period of fourteen days, and then attempted to produce hæmolysins to sheep red cells and precipitins in these animals. They report definite inhibition of hæmolysin and precipitin formation in such animals. Thus in control rabbits, the average maximum titre of the hæmolysin produced was 1 in 1600, this titre being reached about the ninth day, whereas in the experimental animals the average maximum titre was 1 in 140.

The rate of production was also delayed, for this titre was not obtained, on an average, before the fourteenth day. Precipitin formation was similarly influenced, and rats reacted in a similar manner to rabbits. Their conclusion was that "hæmolysin and precipitin formation can be almost completely prevented in animals by saturation with a colloidal dye which is known to be selectively stored by the reticulo-endothelial system."

In their paper they quote the results of Vanucci, who found that, after injections of carmine and Wasserblau, agglutinin formation was decreased, and of Standenath, who, employing chinese ink, found that precipitin formation was increased rather than decreased.

Lacassagne and Paulin (1926) have investigated the effect of destroying or impairing the function of the reticulo-endothelial system by means of polonium, a radio-active body. This they consider more satisfactory than saturating it with an indifferent substance, such as chinese ink or carmine. They subsequently injected the experimental animals with diphtheria and tetanus antitoxin, and found that the average titre of the antitoxin obtained

from such animals did not differ from that obtained from controls. They conclude that their experiment does not bear out the hypothesis that the reticulo-endothelial system is the site of antibody formation.

Simitch (1926), reasoning that the mechanisms of anaphylaxis and immunity are similar, has investigated the effect of the injection of iron oxide and chinese ink on anaphylactic shock. He found no difference in the anaphylactic phenomena between treated animals and controls.

It will be seen that, with the exception of Gay and Clark's experiment, the investigations which have been reported have failed to prove that it is possible to influence antibody formation by previous procedures intended to inhibit the functioning of the reticulo-endothelial system.

Gay and Clark, however, used as a "saturating" agent a colloidal dye that was not used in any of the other experiments, and moreover the results which they report most fully are those of hæmolysin formation against sheep red cells.

It was therefore decided to repeat this part of their experiment, and the present communication reports the results of these investigations.

EXPERIMENTAL.

The conditions under which the experiments were conducted were standardized as far as possible. The trypan blue, used as saturating agent in the first set of experiments, was of pre-war origin, and was supplied to the Director of Veterinary Research in Southern Rhodesia by the late Prof. Ehrlich. The colloidal nature of the dye was first established before using it in the experiments.

The rabbits which were employed as experimental animals were of approximately the same age and weight, and were proved to possess no natural hæmolysins to the sheep cells employed. During the experiment treated and control animals occupied the same out-door run. The sheep cells used in the inoculation of the rabbits, and in the subsequent determination of titre tests, were derived from one animal in order to avoid the possibility of variation in the antigenic value of cells derived from different sheep.

As regards the actual technique, that of Gay and Clark was followed, both as regards the preliminary saturation with trypan blue and subsequent inoculation of sheep red cells.

Details of the experiment are as follows:

Rabbits 1, 2 and 3 were inoculated intraperitoneally daily for a period of fourteen days with 2·5 c.c. of a 1% solution of trypan blue in distilled water. Rabbits 4 and 5 were similarly inoculated, but in this instance 5 c.c. of a 1½% solution of trypan blue were inoculated daily. Throughout this period the animals remained well, and showed evidence of saturation in the intense blue colour of the skin and mucous membranes.

At the end of this period of preliminary treatment the experimental animals, along with normal control rabbits 6, 7, 8, 9, 10 and 11, received intravenously 0·5 c.c. of washed deposited sheep's red cells. The cells were obtained fresh daily, and were washed four times with sterile 0·85% saline in a centrifuge. The hæmolysin content of the serum of each animal was then determined daily for the next fourteen days, and during this period the

experimental animals continued to receive intraperitoneal injections of trypan blue on alternate days.

Rabbit 2 died two days after the third injection of sheep cells, but the remaining animals remained well throughout. The sera of the experimental animals was stained deep blue on every occasion on which it was tested.

The tests for the hæmolytic content of the sera were conducted as follows:

Serum was collected from each animal by bleeding from the ear vein and allowing the blood to clot. The serum was inactivated at 56° C. for thirty minutes. Suitable dilutions were then made with 0·85% saline, and of these dilutions 1 c.c. was used per tube.

Fresh-pooled guinea-pig serum was used as complement, and an excess of this reagent was employed, 0·1 c.c. of a 1 in 4 dilution in 0·85% saline being added per tube.

0·5 c.c. of a 2·5% suspension of packed washed sheep red cells was added to each tube as hæmolytic indicator. Control tubes were included as follows:

In order to exclude the possibility of the trypan blue in the serum acting as a hæmolytic agent, the lowest dilution of serum employed in the test was tested against the sheep red cells suspension in the absence of complement.

In the control animals the serum was similarly tested in the absence of complement.

The possibility of the presence of anti-sheep cell hæmolysin in the complement was similarly excluded by testing the complement, in the necessary dilution, against the red cell suspension in the absence of the inactivated rabbit serum. Controls were invariably negative in the reported results. Results were read after a period of one hour in an incubator at 37° C. The highest dilution showing complete hæmolysis was taken as the titre of the serum, and results were expressed as hæmolytic units per c.c.

The results are given in Table I. This table exhibits an absolute discrepancy from the results of Gay and Clark in that the extent and rate of hæmolysin formation are similar in experimental and control animals. Control rabbit 8 exhibits the poorest response, but the fact that this animal gave birth to young during the experiment may in part at least account for this phenomenon.

In view, then, of the negative nature of the results, it was decided to repeat the experiment, on similar lines, but employing two different brands of trypan blue and lengthening the period of preliminary treatment.

In this experiment three sets of three rabbits each were employed. The rabbits were chosen and looked after as in the previous investigation.

Rabbits 12, 13 and 14 received 5 c.c. of a 1·5% trypan blue solution in 0·85% saline. The trypan blue was the same as that employed in the previous experiment. Rabbits 15, 16 and 17 received 5 c.c. of a 1·5% trypan blue solution in 0·85% saline, the trypan blue in this case being manufactured at Hoechst.

Rabbits 18, 19 and 20 were untreated and served as controls.

The rabbits receiving the trypan blue were given daily intraperitoneal injections for one month. All rabbits then received three injections of 0·5 c.c. washed sheep red cells intravenously, and the hæmolytic titre of the serum then estimated as before for a period of ten days. During this latter period the experimental animals continued to receive daily injections of trypan blue.

TABLE I.—*Titre of Serum on Successive Days. Figures Represent Number of Hæmolytic Units per* 1 *c.c.*

	Days.													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Rabbit 1 (T.B.) .	10	100	200	1250	5000	6000	6000	5000	3000	3000	2000	1500	1250	1250
„ 2 (T.B.) .	20	100	200	800	3000	4000	5000	4000	1500	1250	800	800	800	800
„ 3 (T.B.) .	10	—	—	—	—	—	—	—	—	—	—	—	—	—
„ 4 (T.B.) .	20	100	200	400	1500	3000	4000	6000	5000	3000	3000	3000	1500	1500
„ 5 (T.B.) .	10	100	300	600	1250	2000	4000	4000	5000	2000	2000	1250	1250	1000
„ 6 (C.) . .	20	100	200	1250	3000	5000	6000	5000	5000	2000	1250	1000	800	800
„ 7 (C.) . .	20	100	200	1000	2000	3000	5000	5000	2000	1500	800	800	800	800
„ 8 (C.) . .	0	20	100	200	300	400	600	600	800	800	400	400	400	200
„ 9 (C.) . .	100	600	1250	1500	1500	2000	3000	5000	6000	5000	3000	3000	1500	1250
„ 10 (C.) . .	20	200	800	1250	2000	3000	5000	5000	3000	1500	1500	1500	1250	1250
„ 11 (C.) . .	10	200	600	800	1500	3000	3000	4000	4000	3000	1500	1500	1000	800

T.B. = Trypan blue animal. C. = Control animal.

TABLE II.—*Titre of Serum on Successive Days. Figures Represent Number of Hæmolytic Units per* 1 *c.c.*

	Days.									
	1	2	3	4	5	6	7	8	9	10
Rabbit 12 (T.B.) (Ehrlich) .	100	400	1000	2000	3000	4000	4000	4000	2000	1000
„ 13 (T.B.) „ . .	400	800	2000	2000	4000	4000	3000	3000	1500	800
„ 14 (T.B.) „ . .	50	400	1500	4000	4000	4000	2000	4000	2000	1500
„ 15 (T.B.) (Hoechst) .	100	800	2000	3000	5000	4000	2000	1000	1000	800
„ 16 (T.B.) „ . .	100	800	1000	1000	2000	2000	2000	1000	1000	600
„ 17 (T.B.) „ . .	200	800	2000	2000	4000	5000	4000	2000	1000	800
„ 18 (C.). . . .	400	1000	3000	4000	4000	2000	1000	1000	800	600
„ 19 (C.) . . .	200	800	1500	2000	2000	2000	1500	1000	800	800
„ 20 (C.) . . .	100	600	1000	2000	2000	2000	1000	1000	800	800

T.B. = Trypan blue animal. C. = Control animal.

Twenty-four hours were allowed to elapse between the administration of trypan blue and the taking of blood for hæmolytic tests.

The sheep red cells used were again derived from one animal throughout the experiment, but the donor was a different animal from that employed in the first experiment.

An attempt was made to estimate roughly the concentration of trypan blue present in the sera of the experimental animals during the period when the content of hæmolytic amboceptor was being tested. Trypan blue when added to serum loses the dark blue colour it exhibits when dissolved in aqueous solution, and becomes bluish-green. Accordingly it was necessary to make various dilutions of an aqueous solution of trypan blue in rabbits' serum, in order to estimate even roughly the degree to which it was present in the sera of the "saturated" animals.

A comparison was made between tubes containing known concentrations of trypan blue and the serum of these animals on the first, fourth, eighth and twelfth day after the last injection of sheep cells. It was found that the concentration of the dye remained remarkably constant, although slight variation in content existed between different animals. The average concentration lay between 1 in 7500 and 1 in 10,000, and it can be assumed that this represents the minimum concentration of the dye throughout, since the sera were tested at a twenty-four-hours' interval from the last injection of trypan blue.

As regards the tests for the hæmolytic amboceptor content, these were conducted as in the previous experiment. The results of this second experiment are given in Table II, and it will be seen that they confirm the results obtained in the previous one. It would thus appear that saturation with trypan blue in the doses employed has no influence on the subsequent formation of hæmolysins in response to the introduction of sheep red cells.

It is difficult to correlate these results with those of Gay and Clark, particularly as their communication was a preliminary one, and no details are given as to the number of animals employed, nor as to the dosage and strength of the trypan blue used. It can be surmised that, as regards strength of solution, that which was employed by them could not have been much more concentrated than those used in the present investigation, since it was found that with both varieties a 1·5% solution was practically a saturated solution.

While again it is difficult to determine what constitutes a "saturated" animal in their estimation, there is evidence in the present experiment that the dye was present in considerable concentration in the blood-stream. An autopsy which was performed on the animal which died in the first series showed that the skin, subcutaneous tissue, mucous membranes, serous membranes, liver, spleen and kidneys were stained intensely blue, all of which points to the dye having been taken up by the cells of these tissues.

Unstained sections from the liver showed the Kupffer cells to be dark blue, which warrants the assumption that the dye was in part at least actually taken up by the cells of the reticulo-endothelial system.

The results obtained, then, are not in favour of the hypothesis that it is possible to influence hæmolysin formation by means of the previous saturation of the animal by an indifferent colloidal dye.

In this they correspond with those of Bieling, Lacassagne and Paulin, and

Simitch, who came to the conclusion that antibody formation was not affected by measures intended to "block" or destroy the reticulo-endothelial system.

SUMMARY.

Saturation of rabbits, by daily intraperitoneal injections of trypan blue for periods up to one month, had no effect on subsequent hæmolysin formation in these animals, in response to intravenous injections of sheep red cells.

I desire to express my thanks to Mr. Bevan, Director of Veterinary Research, Southern Rhodesia, for supplies of trypan blue, and for providing the sheep from which the cells used in the experiment were taken.

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OBSERVATIONS ON THE ALTERED RENAL THRESHOLD FOR SUGAR IN INSULIN-TREATED DIABETICS.

I. M. RABINOWITCH.

From the Department of Metabolism, the Montreal General Hospital, Montreal, Canada.

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In a recent communication (1924) the writer reported two cases of diabetes in which the renal thresholds for glucose were observed to have altered a very short time after the institution of insulin treatment. During the early periods of observation the urines were found to contain sugar when the blood-sugars ranged between 0·16 and 0·18%. Shortly after, while the diets were still being regulated, no sugar was excreted in the urines in spite of the fact that the blood-sugars ranged between 0·20 and 0·24%. In both of these cases hyperglycæmia was accompanied by polyuria. Neither of these individuals had albuminuria, nor were the cases of long duration—two of the conditions in which a raised renal threshold for glucose is generally met with in diabetics. Observations made upon these individuals subsequently at regular monthly intervals for a period of over two years showed that the condition persisted.

This absence of glycosuria in the presence of hyperglycæmia is a problem. Such patients, though excreting no sugar in the urine, must, on account of the hyperglycæmia, be looked upon as active diabetics, and therefore exposed to the various complications of the disease. As a matter of fact, one of these patients developed a carbuncle. One disturbing factor is that the absence of sugar in the urine, usually a favourable sign, ceases to be an index of progress in such cases. The essential data of one of these cases previously reported are reproduced here:

Case 1.—6742/22, B. M—, a girl, aged thirteen, was admitted to the Montreal General Hospital on December 2nd, 1922, practically in coma. Under insulin treatment she recovered and subsequently did not require any insulin. She was discharged from the hospital December 21st, 1922. The blood and urinary findings were then normal. Blood analyses made every two weeks thereafter showed that the post-absorptive blood-sugars were persistently normal until May 19th, 1923, when she developed a mild respiratory infection, not unlike influenza in nature. Following this infection, glycosuria was found at frequent intervals, and on July 31st, 1923, it was necessary to again institute insulin treatment.

Because of the unsatisfactory progress which the patient was making at home she was readmitted for observation on December 26th, 1923. Until January 10th, 1924, there appeared to be nothing peculiar about the results noted, and for this reason these are not recorded. The threshold level appeared to be that of the average individual. At a blood-sugar of 0·175% glycosuria was noted. In Table I are recorded the essential findings from that date to demonstrate the phenomenon bearing on the subject of this communication.

TABLE I.

Date.	Urine.					Blood: Sugar per cent.
	Volume.	Sugar per cent., grammes.	Acetone.	Tit. acid, c.c. N/10 NH_3 per day.	NH_3, grammes per day.	
January						
10	2700	0	0	842	1·06	0·217
11	2800	0	0	739	0·91	—
12	2600	0	0	728	0·88	—
13	2800	0	Very faint trace	795	0·99	0·232
14	2400	0	0	778	0·88	—
15	3000	0	0	816	0·97	0·217
16	2700	0	0	799	0·97	—
17	2160	0	0	803	0·93	—
18	2360	0	0	—	—	0·244
19	3000	0	0	744	1·02	—
20	2700	0	0	875	1·01	0·212
21	3300	0	0	972	0·55	—

TABLE II.

No.	Hospital No.	Age (years).	Threshold studies.				Time intervals (days).	Renal efficiency studies.					Urea concentration factor.	Water test.
			Glycosuria.		No glycosuria.			Blood urea nitrogen, mgms. per 100 c.c.		Blood creatinine, mgms. per 100 c.c.	Urine urea concentration (%).			
			Date.	Blood-sugar (%).	Date.	Blood-sugar (%).		Before giving urea.	After giving urea.		After giving urea.			
											1 hour (%).	2 hours (%).		
1	6742/22	13	Dec. 26/23	0·175	Jan. 10/24	0·333	15	14	25	1·00	2 86	2·17	52	Negative.
2	H. G.	22	Jan. 16/24	0·181	Mar. 18/24	0·232	31	20	30	1·32	2·62	2·31	43	„
3	A. T.	17	Mar. 16/24	0·191	Oct. 20/25	0·285	218	18	32	1·14	2·84	2·92	54	Positive.
4	3678/23	12	Sept. 3/24	0·169	Sept. 10/24	0·333	8	20	29	1·25	3·12	3·24	62	Negative.
5	N. O. R	19	Oct. 6/24	0 194	Feb. 9/26	0·312	491	18	32	1·54	3 45	3 60	67	Positive.
6	5407/24	54	Oct. 5/24	0 181	Jan. 7/25	0·356	74	22	30	1·53	2 07	2·14	38	Negative.
7	5554/23	29	Nov. 22/24	0·208	April 3/25	0·256	132	20	29	1·26	3 60	3·32	63	„
8	L. A D.	49	Mar. 23/25	0·201	Aug. 26/25	0·312	156	21	25	1·51	2·34	2·76	56	„
9	2526/25	10	July 9/25	0·184	Dec. 29/25	0·221	173	21	33	1·32	2·72	3·51	61	„
10	C. A. M.	14	Aug. 28/25	0·177	Sept. 21/25	0·250	24	16	20	1·24	2·34	2·06	53	„
11	S. I. F.	21	Sept. 5/25	0·168	Dec. 4/25	0·217	70	21	27	1 07	3·20	3 11	61	„
12	A. E.	6	Nov. 6/25	0·162	Jan. 22/26	0·400	61	11	No other studies made.				—	—
13	5921/25	42	Jan. 5/26	0·192	Mar. 9/26	0 241	63	20	25	1·43	2·11	2·62	53	Negative.
14	1463/26	28	April 9/26	0·169	May 8/26	0 333	29	18	29	1·62	2·04	2·21	44	Positive.

The polyuria and hyperglycæmia in the absence of glycosuria are noted here. There was no acidosis. Clinically, the patient looked and felt very well, and was gradually gaining weight. Since then twelve other such cases have been observed and an effort has been made to determine the underlying nature of this condition (Table II).

It is generally recognized that two common causes of alteration of the renal threshold for glucose in diabetes, other than nephritis, are indiscretion in diet and a chronic condition of the disease. Of the remaining twelve subjects, it is definitely known that a number did not follow their instructions—for a time following the idea of "more insulin, more food." It was during these periods that the high renal thresholds were observed. When, however, the diets were readjusted and the patients were following instructions, the renal threshold failed to return to the normal.

The condition also appears to differ from that found in cases of long duration, in the rapid onset. Some of these alterations are known to have developed within two months of the institution of insulin treatment. In five of these cases there was an accompanying polyuria. This does not usually accompany the raised threshold generally observed in chronic diabetes. None of these individuals had albuminuria. Eight of the fourteen subjects were under twenty-one years of age. It is interesting here to note that this phenomenon was observed in one patient (No. 11) with a condition which is usually accompanied by a lower renal threshold for glucose, namely, pregnancy.

In view of the possibility that the raised threshold may be the result of impairment in renal efficiency, observations were made with reference to the latter, and the object of this communication is to record the results of the studies made.

In each case, with the exception of No. 12, the following data were obtained: Blood-urea nitrogen, blood creatinine, urine-urea concentration following the ingestion of urea, the urea concentration factor and the "water test." The combined results are recorded in the accompanying Table II. In each case are also recorded the age of the subject, the date of the last observation when the relationship between hyperglycæmia and glycosuria was found to be normal, and the threshold value at the time; the date when the raised threshold was first observed, and the degree of hyperglycæmia at the time.

DISCUSSION OF RESULTS.

The marked hyperglycæmias are obvious. The greatest degree (0·4%) was noted in the youngest subject (No. 12). Unfortunately in this case no renal efficiency tests were made with the exception of the blood-urea estimation. As stated before, however, there was no albuminuria. It will be noted that so far as excretion of nitrogenous substances is concerned, there appeared to be no evidence of impairment in renal efficiency, as judged by the methods of examination presently available. In three cases—Nos. 3, 5 and 14—the "water tests" suggested impairment in water-excreting power. It is interesting here to note that these three subjects had what is now generally

known, for want of a better term, as "insulin œdema." These subjects, following the institution of insulin treatment, gain weight more rapidly than can be accounted for by increased body-tissue. The gain in weight is apparently due to retention of large quantities of water, since, when insulin is discontinued, a rapid loss of body-weight follows. This loss of body-weight is accompanied by polyuria, takes place without any other alteration in the diet, and is not accompanied by glycosuria. Though many other "œdema" cases have been observed, only the three recorded presented the raised threshold phenomenon.

In view of the above findings, it appears that impairment in renal efficiency does not account for this phenomenon. No explanation is offered. These cases are reported merely to emphasize the fact that for certain individuals, following the institution of insulin treatment, the absence of sugar in the urine ceases to be a reliable index of progress. Fortunately these individuals represent a very small proportion of the total number of diabetics requiring insulin.

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METHOD FOR DETERMINING THE ANTISCORBUTIC VALUE OF A FOODSTUFF BY MEANS OF HISTOLOGICAL EXAMINATION OF THE TEETH OF YOUNG GUINEA-PIGS.

AXEL HÖJER.

From the Institute for Pathology and the Medico-Chemical Institute, Lund, Sweden.

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HOLST, in 1907, carried out experiments on young guinea-pigs in order to determine the antiscorbutic value of foodstuffs. As a unit he took the least dose that protects young guinea-pigs, weight 150–300 gm., from manifest signs of scurvy. This dose he called the minimum protective dose. Unfortunately, even after a period of six months on a given diet, there is no certainty that the guinea-pig in question may not at some period present scorbutic hæmorrhages. Sherman and others, in 1922, therefore proposed to limit the experimental time to 70 or 90 days and then to make a macroscopic post-mortem examination, to make sure that there were no internal hæmorrhages or other signs of scurvy. Sherman also introduced the rating of the scorbutic signs in animals which were not fully protected, and thus he estimated the dose of antiscorbutic given in quarters of the fully protective dose. Even with these improvements of

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FIG. 1.

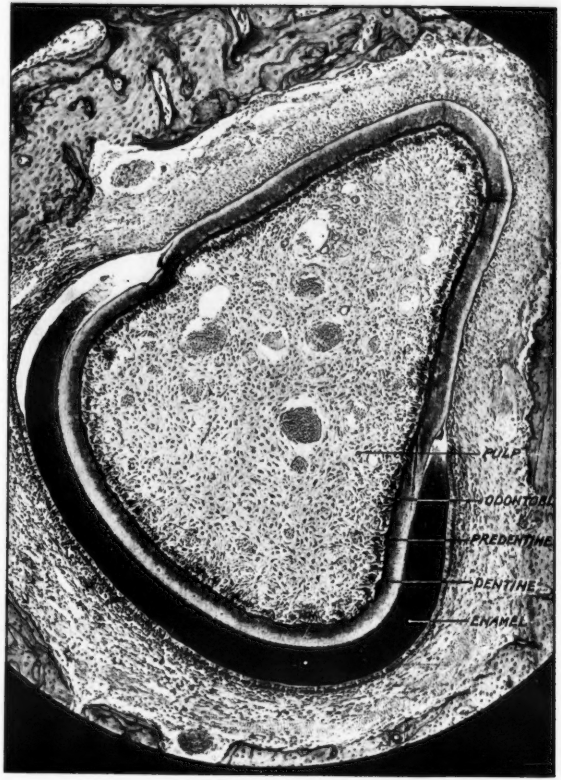

FIG. 2.

Höjer.

Sherman's this method of determining the fully protective dose is very inaccurate, and often gives variations of 100% or more.

The discovery in 1916 by Jackson and Moore of scorbutic changes in the teeth, their recognition as being very early manifestations of scurvy by Zilva in 1919, and their detailed description as specific by me in 1924, form the basis of another method, or rather, a modification of the former method for measuring the antiscorbutic value of foods.

THE TECHNIQUE.

As previously, young guinea-pigs from a certain day on are given a basal diet free from antiscorbutic, but otherwise complete, and to this diet are added quantitatively daily doses of the juice to be examined. For a sharp determination of the fully protective dose it is advisable to have several animals on different doses. In addition there ought to be two control animals on the basal diet alone, and two on the basal diet with a fully protective dose of a known antiscorbutic. After a period of 10–14 days all the animals are killed. The one half of the lower jaw is taken out and decalcified in a 5% tri-chloracetic acid solution. It is then embedded and sectioned after one week. The section is made through the foremost molar at right angles to the longitudinal axis of the jaw. Some sections are stained with hæmatoxylin-eosin, and others with tri-oxyhæmatin according to the method of Hansen.

EXAMINATION OF THE SECTIONS.

The interest is concentrated on the cross-section of the incisor root (Fig. 1). When the antiscorbutic dose given has been fully protective—that is, when there is no scurvy—there appears, when stained with hæmatoxylin-eosin, a broad, uniformly stained pink layer of dentine, a white, uncalcified predentine, a layer of long, slender parallel odontoblasts, and a pulp of small, star-shaped cells. Stained according to Hansen's method, the dental fibrils of the dentine and predentine are all sparkling red. When the antiscorbutic dose given is somewhat lower—equal to 0·9 of the fully protective dose—there is a marked difference. The odontoblast layer on the larger pole is distinctly narrower. When the antiscorbutic dose given is equal to 0·8 of the fully protective dose, the uniformity of the dentine staining is gone. Between the old and the freshly formed dentine there is a sharp outline. The freshly made dentine has a lighter colour and less collagen.

When no antiscorbutic is given (Fig. 2) there is quite another picture. The dentine is rather thin. The predentine stains with hæmatoxylin-eosin very darkly, because it is calcified. With Hansen's method, on the contrary, it is quite white, being deprived of its collagen. The odontoblasts on the larger pole do not exist any longer as a continuous layer. They seem to be on a disordered march into the hyperæmic pulp, and do not deserve the name of odontoblasts any longer, as they are not forming dentine, but bone in the form of an irregular network. The odontoblasts have become osteoblasts.

When the daily antiscorbutic dose given is lowered from 0·8 to 0·1, the character of the odontoblasts also gradually changes. This is seen both in

their shape and arrangement and in their products, dentine and predentine. These pictures form middle stages between those just described.

Thus it is obvious whether a fully protective dose is given or not. The error for this estimation does not exceed 10%. For purely scientific work it is advisable to settle the least antiscorbutic dose, that is the least fully protective, because it is most sharply determined.

For practical hygienic purposes an estimate may be made in tenths of the protective dose. Even if this estimation should be somewhat uncertain, it is at least just as good as that of Sherman, and it requires much less time. I do not pretend that this method is perfectly simple. It requires a close study of the different stages, especially because the pictures diverge more distinctly when the animals live a little longer. Pictures and descriptions illustrating these points will be found in my work of 1924, together with a titrated series for comparison. It must also be remembered that single animals may diverge a good deal from the others of the same group. With these reservations I put forward the following scheme:

TABLE I.—*Determination of the Antiscorbutic Value of Food by Höjer's Method.*

	Part of protective dose.
1. Dentine of normal size; its inner and outer layer uniformly coloured; predentine regular, uncalcified; dentine and predentine holding collagen.	
a. Odontoblasts long, slender, parallel, of equal height.	1·0 or more.
b. Odontoblasts partly shorter	0·9
2. Dentine very thin, uniformly coloured; odontoblasts parallel, short . . (The degree of osteoporosis, hyperæmia and collagen permit the distinguishing of different stages, but not very sharply. Those animals whose dentine formation seems to have been arrested are therefore excluded.)	0·8–0·3
3. Dentine in inner and outer layer differently coloured.	
a. Odontoblasts on the larger pole of the incisor cross-section short, parallel	0·8–0·3
(*α*) The Tomes's canals going parallel through a normal predentine .	0·8
(*β*) The uncalcified predentine defective; formation of network-bone beginning	0·7
(*γ*) Uncalcified predentine lacking	0·7–0·5
(*δ*) Network bone-formation greater	0·4
(*ε*) Tomes's canals in the old dentine widened	0·3
b. Odontoblasts on the larger pole of the incisor cross-section no longer in continuous layer	(0·3–) 0·2–0·0
(*α*) Tomes's canals in the outer layer of the new bone	0·2
(*β*) No Tomes's canals in the new bone; osteoporosis and hyperæmia well developed	0·1
(*γ*) The old odontoblasts in greatest disorder; osteoporosis and hyperæmia very evident	0·0

In clinical work I have found this method useful. In February, 1925, I examined the antiscorbutic value of milk sold as baby milk to the children's hospitals in Stockholm. 100 c.c. raw milk were given to each of four guinea-pigs. The tooth pictures represented 0·1 to 0·2 of a fully protective dose. It was concluded that 500 to 1000 c.c. for guinea-pigs, or 18 to 36 pints in the case of children, represented the fully protective dose of this winter milk. On the contrary, in experiments conducted this summer, when I tried to get rid

of the antiscorbutic in the milk through treatment with air at 100° (Celsius) for one hour, there remained so much of the antiscorbutic in the milk that the fully protective dose was only 100 to 200 c.c. for guinea-pigs, or 4 to 7 pints for children. You may thus in the winter-time have milk deprived of vitamin C, as tested by the above basal diet, but not in the summer.

As another example of what this scheme might yield I give the result of a control titration of a strong antiscorbutic, the Northern wild cloud berries, which have as high an antiscorbutic value as orange-juice.

TABLE II.—*Determination of the Antiscorbutic "Protective Dose" by Höjer's Method.*

Animal No.	Juice given (c.c.).	Juice given as part of protective dose.	Juice determined as part of protective dose.
1–3	0	0	0
4–6	0·3	0·1	0·2
7–9	0·6	<0·2	0·1
10–12	0·9	<0·3	0·3
13–15	1·2	0·35	0·3
16–18	1·5	>0·4	0·4
19–21	1·8	0·5	0·5
22–24	2·1	0·6	0·4 *–0·7*
25–27	2·4	0·7	*0·35*–0·7
28–30	2·7	<0·8	*0·4* –0·7
31–33	3·0	<0·9	0·9
34–35	3·5	1·0	1·0 or more
36–37	4·0	>1·1	1·0 „
38–39	4·5	1·3	1·0 „
40	5·0	1·5	1·0 „

The harmony is fairly good. In each group there were generally three animals, which were each given a daily dose of juice varying from 5 c.c. to none. It was found that 3·5 c.c. just represented the fully protective dose. One animal in this group was fully protected, the other was not. Thus it was easy to tell which part of the protective dose the doses given represented. Now compare these figures with those derived from the estimation of the sections (Table II, cols. 3 and 4). In the experiments dealing with the smaller doses of antiscorbutic and again in those affording complete protection there is close agreement. With seven- and eight-tenths of the protective dose there is a greater error than with one-tenth, and the error is rather great (50%). The testing is indeed difficult in that part of the scheme which extends from 0·4 to 0·7 or sometimes 0·8. Special precaution is evidently needed for this part of the scheme. The method may, however, be made sharper by letting the animals live longer than two weeks.

Naturally a biological method cannot afford the accuracy or the simpleness of a chemical one. It is indeed the lack of a chemical method for the determination of the antiscorbutic value that has called in experimental

pathology as a temporary aid to the physiologist. As a biological method, and in comparison with that hitherto used, my method has the following advantages: First, it is accurate in fixing the fully protective dose. This is of value particularly in physiological work. Secondly, the method requires only three weeks instead of three months. This is an essential factor in hygienic work. Lastly, being shorter, it is also more economical than the older methods.

REFERENCE.

The authors referred to will be found in the following paper:
HÖJER, A.—(1924) *Acta prædiat.*, **3** (suppl.).

THE EFFECT OF HIGH PROTEIN DIET ON RENAL FUNCTION.

H. MACLEAN, J. FOREST SMITH AND A. L. URQUHART.

From the Medical Unit Laboratories, St. Thomas's Hospital, London.

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THE experiments described in the present paper were carried out with a view to determining the effect of high and excessive protein intake on renal function. Though it is generally accepted as a clinical fact that ingestion of protein is detrimental to individuals suffering from renal disease, there does not seem to be any definite experimental evidence available in support of this contention; indeed, the clinical observations on which this belief is based seem in many cases to be entirely misleading. The question as to whether or not protein under certain conditions is injurious to the kidneys is of very great practical importance for the rational treatment of renal disease, and the following investigation is an attempt to throw some light on this problem.

Within recent years many observers have found that an increase of protein in the diet of such animals as rats and rabbits often resulted in marked changes, both in the function and structure of the kidneys. Newburgh (1919) fed rabbits on a mixture of egg-white, casein and soya bean, and produced changes which he describes as congestion of the kidneys; some definite tubular lesions were also present. On a diet of egg-white, nephritis is claimed to have been produced in a very short time. With casein the results were somewhat different, for when the daily ration of casein did not exceed 15 gm. no renal changes were found, but when the ration was increased to 30 gm. daily, then very marked renal lesions soon manifested themselves. In all these experiments green vegetables were given two or three times weekly in addition to the protein. In a later communication, Newburgh and Clarkson

1922, 1923) claimed to have produced nephritis accompanied by definite arterio-sclerotic changes in rabbits fed on lean dried meat with greens. Definite evidence of a renal lesion was present in about six weeks after the commencement of this diet. Some of the urines passed were definitely acid, and thus differed in reaction from the normal urine of the rabbit. On the other hand, several urines remained alkaline throughout the experiment, but the reaction of the urine made no difference whatever to the onset of nephritis, for renal changes were equally marked, whether the urine passed was acid or alkaline. The general conclusion arrived at was that an acid urine *per se* did not produce nephritis in the rabbit, but that it might lead to some albuminuria. Frandsen (1924) showed that rabbits suffering from toxic renal lesions produced by cantharides or potassium bichromate became uræmic when fed on a diet very rich in nitrogen.

This detrimental effect of protein food on the kidneys has not, however, been accepted by all observers. Osborne and Mendel (1924) found that when rats received high protein diet, some hypertrophy of the kidneys was produced, but no microscopical evidence of damage could be ascertained. This observation was confirmed in subsequent work by Osborne, Mendel, Park and Winternitz (1925), who showed that the hypertrophy produced by excessive protein intake was equivalent to about 50% of the normal kidney weight, and that the kidneys returned to normal size when the nitrogen intake was reduced. No cardiovascular changes were present. Reader and Drummond (1925) fed rats on diets containing 40 or 50 to 90% protein with cod-liver oil, yeast and lemon-juice, but found no evidence of any renal damage, though definite hypertrophy of the kidneys was present. Jackson (1925) also came to the conclusion that nephritis was not produced in rats by feeding on high protein diet over a considerable period.

It is interesting to note that the majority of the experiments showing no evidence of a deleterious action of protein on renal function were carried out on rats, while the experiments of Newburgh and his colleagues, in which high protein diet was claimed to produce nephritis were done on rabbits. It is obvious that the rabbit, being an herbivorous animal, would be more prone to be affected by a high protein intake than a carnivorous animal like the rat. In both animals, also, the amount of vitamins ingested during the experimental period must have played a very important part.

For the purposes of our investigation, it was thought advisable to use an experimental animal that did not normally consume very much protein food, and for this reason all our observations were carried out on rabbits. It was also probable, in the light of the work just described, that rabbits might be more susceptible than rats to renal changes arising from excessive nitrogen intake. Though only a few typical experiments are described here, the work has extended over two years, and the various results have been confirmed several times.

DESCRIPTION OF DIETS USED.

A "normal" diet and a "high protein" diet were used.

The "*normal diet*" consisted of bran 2 parts, oats 1 part, 2 leaves of cabbage per day.

The average analysis of this mixture gave protein 11·5%, carbohydrate 60%.

The "*high protein diet*" consisted of a cake which contained bran 4000 gm., casein 8000 gm., salt 120 gm., gelatine 300 gm.

The casein was ground to a powder by means of a fine mincing-machine. The gelatine was dissolved in hot water and a thick paste made of all the constituents. This was baked and quantities of it fed to the animals. The average analysis of this cake was: protein, 60%; carbohydrate, 12%.

The animals were given as much as they wished to eat of these diets. The "high protein diet" was very well taken, and seemed to be relished quite as much as the ordinary mixture of oats and bran.

DETERMINATION OF RENAL FUNCTION WHEN FED ON NORMAL DIET.

The concentration of blood-urea present at different periods while the animals were being fed on normal diet (bran, oats, and 2 leaves of cabbage per day) was determined; also the daily amount of urine secreted and the concentration of urea in the urine. In addition, the urine was examined periodically for evidence of albumin and casts; no pathological constituents of this nature were ever found while normal diet was in use. The first experiment was carried out on 16 rabbits, and it was found that the blood-urea figures lay between 22 and 50 mgm. per 100 c.c. blood. This agrees with the observation of Newburgh (1919), who states that the average blood-urea of a rabbit on normal diet is 31 mg. per 100 c.c. The urine was persistently alkaline, and the concentration of urea varied between 1·4 and just over 2%. The following figures (Table I) indicate the variations in blood and urine-urea found in 6 of these rabbits when examined over long periods:

TABLE I.

	Lowest readings of blood-urea.			Highest readings of blood-urea.			
No.	Volume of urine in 24 hours.	Urine-urea %.	Blood-urea, mgm. per 100 c.c.	Volume of urine in 24 hours (c.c.)	Urine-urea %.	Blood-urea, mgm. per 100 c.c.	Period under observation (months).
1	194	1·4	23	140	1·55	31	5
2	190	2·1	25	138	1·5	29	5
3	250	1·8	27	170	1·85	37	5
4	212	1·8	24	—	1·9	31	5
5	120	1·5	25	132	1·7	40	12
6	148	2·15	22	56	2·0	50	12

During this period of observation two animals died, one from snuffles and the other from an abscess of the groin. None of these sixteen rabbits showed any signs of nephritis, and there were no changes found post mortem in the kidneys of the two rabbits that died. Having settled the average figures for blood- and urine-urea obtained on normal diet, experiments with "high protein diets" were next carried out.

EFFECT OF HIGH PROTEIN DIET PLUS GREEN FOOD ON RENAL FUNCTION.

The diet given was the cake already described, containing an average of 60% protein. In addition to this, each rabbit received two leaves of cabbage per day. The effect of this diet was very marked. The blood-urea rose to about 100 mgm. per 100 c.c. and the urine-urea reached the high figure of 6% or over. Even with this marked change in urea concentration there was no albuminuria, and no casts appeared in the urine. The general condition of the animals was good and the average weight increased somewhat. At the end of a month or so the blood-urea gradually fell until it reached the normal figure of about 30 mgm. per 100 c.c. This was accompanied by a decrease in the concentration of urea in the urine, but the volume of urine passed was increased to some extent. Specimens of the figures obtained are given in Table II. The period of observation in these cases was about one month.

TABLE II.—(a) *Immediate Effect of High-Protein Diet plus Cabbage.*

No.	Volume of urine in 24 hours.	Reaction.	Urea %.	Blood-urea in mgm. per 100 c.c.
1	—	—	6·1	119
2	82	Acid	6·0	91
3	—	—	5·25	112
4	130	Alkaline	6·1	112
5	140	—	7·0	105
6	—	—	6·8	115

(b) *Later Effects of this Diet.*

No.	Volume of urine in 24 hours.	Reaction.	Urea %.	Blood-urea in mgm. per 100 c.c.
1	210	Neutral	2·75	33
2	200	,,	2·25	32
3	194	,,	2·3	35
4	215	,,	2·1	30
5	170	Alkaline	2·0	30
6	210	Neutral	1·75	35

These results indicate that the early increase of urea in the blood and urine is not associated with any renal damage. The comparative rapidity with which the kidneys adapt themselves to the large protein intake is somewhat remarkable, for in a few weeks no evidence of the greatly increased protein intake was obtainable by examination of the blood or urine. These experiments do not support the contention of Newburgh and his colleagues that ingestion of large amounts of protein produces nephritis in rabbits. No evidence whatever of nephritis could be obtained in any of our rabbits when a little cabbage was given with the high-protein diet. It was noticed that this diet tended to reduce the alkalinity of the urine. In the majority of cases the urine became neutral, and in one case definitely acid without any albumin occurring.

One objection to the above experiment might be advanced. It might be argued that a much longer continuance of this high-protein diet would ultimately result in definite renal changes despite the fact that no such changes were in evidence after one month. To test this five rabbits were put on this diet; three of them were observed for a period of 109 days, and the other two for 123 and 152 days respectively. All these rabbits gained weight; those on the diet of the shorter period gained an average of 300 gm., while that on the longer period of 152 days gained 930 gm. None of these animals showed albumin or casts in the urine or any other evidence of nephritis.

Since no detrimental effect on renal function could be produced by high protein diet plus green food, even when this diet was continued for over 5 months, the next experiment was arranged to determine the effect of high protein diet in the absence of green food.

EFFECT OF HIGH PROTEIN DIET WITHOUT GREEN FOOD ON RENAL FUNCTION.

Two rabbits were put on this diet, and in 18 and 23 days respectively they developed nephritis. Albumin and casts were present in abundance in the urine. Both rabbits showed a marked loss of weight, one 440 gm., the other 690 gm.

After the nephritis condition had been present in the case of the first rabbit for 12 and in the second for 23 days, the former was given two leaves of cabbage per day. After 18 days the passage of albumin and casts completely ceased. This rabbit has been under observation for nine months since the nephritis attack, and has remained on high protein diet with green food in perfect health. The second rabbit was killed in order to observe the condition of the organs.

In another experiment five rabbits were put on the high protein diet without green food, water being given as before. One animal was exposed in its cage to sunlight, and did not develop nephritis until after 47 days. The others were kept in the animal house, and they developed nephritis as indicated by albumin and casts in the urine in 12, 14 and 24 days.

The following table shows the time elapsing between the removal of the green food and the appearance of albuminuria and casts in the case of six other rabbits on a high protein diet:

TABLE III.

No.	Number of days to produce nephritis.
1	28
2	23
3	23
4	19
5	18
6	23

PATHOLOGICAL FINDINGS *POST MORTEM* OF RABBITS THAT HAD DEVELOPED NEPHRITIS ON HIGH PROTEIN DIET MINUS GREEN FOOD.

The animals that were fed on high protein diet with green food all gained weight, but when green food was withheld a marked loss of weight was noticed in all cases as described. At the post-mortem all the animals were thin and showed scanty subcutaneous and subperitoneal fat.

Table IV shows the weight of the organs of these nephritic animals fed on a high protein diet. No. 1 is given as a normal average control.

TABLE IV.

No.	Weight on green food.	Weight without green food.	Spleen (gm.).	Heart (gm.).	Liver (gm.).	Kidney, right (gm.).	Kidney, left (gm.).	Duration of nephritis.
1	3650	—	2·3	8·7	138·7	9·0	9·1	—
2	3320	2200	0·6	7·0	67·2	10·6	11·0	15 days.
3	3450	3000	1·3	9·0	96·0	12·6	12·3	6 „
4	3050	2040	0·8	7·4	63·9	10·2	9·6	46 „
5	3700	1910	0·6	6·5	56·0	11·0	10·6	61 „
6	2700	—	—	6·7	94·7	8·8	8·6	12 „

It will be seen that the weight of the spleen, liver and heart was considerably reduced when the nephritic condition had existed for some time, and this corresponded with the marked loss of total weight in the animals. The actual weights of the kidneys did not show a reduction; indeed, in certain cases it would appear that the kidneys had increased in weight.

REPORT ON MICROSCOPICAL FINDINGS IN ORGANS OF THIS GROUP.

The spleen of these rabbits showed dilated sinuses. In many of them more or less active phagocytosis of red cells was present. In three rabbits there was a marked deposit of free iron, the yellowish iron pigment being a striking feature of the sections.

The liver showed no microscopical changes, either in the production of excess of fibrous tissue or in degeneration of the cells.

The kidneys in every case showed alteration from the normal, the change being seen chiefly in the convoluted tubules. It was found that the tubules were dilated, some almost to cyst-like spaces lined by a flat epithelium. The straight tubules were not so greatly affected, but in many cases casts were present in the lumen. Fatty degeneration was present to some extent in the renal tubular epithelium, but was most marked in the areas of the convoluted tubules. The Bowman capsules and glomerular tufts appeared to be unaffected and no changes were observed in the renal vessels.

In those cases where the nephritis was of short duration the dilatation of the tubules was patchy in its distribution. As the nephritic condition persisted the tubular lesion apparently spread through the cortex. In no instance was any chronic inflammatory reaction noted.

In the rabbits showing a marked free iron reaction in the spleen, a small amount of iron was present in the kidneys.

Rabbits kept under these conditions always died within a short time after development of nephritis and rapid loss of weight was noted, so that the keeping of such animals over periods which might have produced chronic interstitial changes was found to be impossible. On the other hand, as already indicated, the condition of nephritis produced by lack of green food was found to be recoverable when the necessary green food was supplied.

Since the importance of green food in the diet was now definitely established, it was considered advisable to ascertain whether nephritic changes could be produced by ordinary diet in the absence of green food. This diet is the usual one on which rabbits are fed in our department.

EFFECT OF ORDINARY DIET, BUT WITHOUT GREEN FOOD, ON RENAL FUNCTION.

Two rabbits were fed on bran and oats with a small amount of green food over a period of three months, during which both animals put on weight to the extent of 500 and 400 gm. During this time the urinary function was normal.

Green food was removed from the diet and water substituted. Within 24 days both animals developed nephritis. The urine became acid and both albumin and casts were present. One rabbit died 7 months afterwards, its weight dropping from 3500 gm. to 1980 gm. in 187 days. This loss of weight was gradual at first, becoming very rapid a week or so before death, when the animal appeared emaciated and ill. During these 187 days albumin and casts were present in the urine.

At the post-mortem, made within an hour after death, marked general emaciation was found, little normal fat being seen. On microscopical examination the *spleen* showed marked dilatation with the formation of sinuses; active phagocytosis of red cells was observed. The free iron reaction was very definite. The *liver*-cells appeared small and closely packed. No fatty or fibrous change was seen. The *heart* was small and the vessels showed no evidence of degenerative change. The *kidneys* exhibited very marked dilatation, especially in the superficial cortical areas. The lining epithelium of these tubules was flattened. Fatty changes in the general renal epithelium were well marked. Casts were present in abundance. No change was noted in the blood-vessels, or in the glomerular tufts or Bowman capsules. There was no suggestion of any chronic inflammatory lesion.

The other rabbit weighed 3650 gm. at the start of the nephritis and in 18 days it lost 500 gm. At this stage, green food which had been boiled for one hour was added to the diet. In 24 days the albuminuria had decreased very much and the casts had entirely disappeared from the urine. The weight continued at 3100 gm., but unfortunately death occurred 11 days later from sepsis affecting the ears, eyes and nose. The following are the post-mortem findings: The *spleen* showed practically no changes; the *liver*-cells showed cloudy swelling, but no fatty degeneration; the *kidneys* showed a few scattered patches of dilatation in the cortical area with flattened epithelium.

Marked fatty degeneration was present in the tubular epithelium; no glomerular changes or any evidence of chronic inflammation were found.

Table V shows well the effect of absence of green food from the normal diet in rabbits. Rabbits Nos. 1 and 2 show the results of feeding on normal diet plus green food. Nos. 3 and 4 show the results of a similar diet but without green food. In the absence of green food nephritis developed in these two rabbits in 24 days.

TABLE V.

No.	Weight of rabbit.	Weight of spleen (gm.).	Weight of liver (gm.).	Weight of heart (gm.).	Weight of right kidney (gm.).	Weight of left kidney (gm.).
1	4005	6·1	151	13·3	15·4	14·7
2	3270	5·8	116·6	9·7	14·6	14·6
3	3500–1980	0·5	65·8	6·5	6·0	6·5
4	3600–3100	1·2	75·9	9·5	8·1	8·6

Other experiments substantiated the above results, and showed conclusively that the absence of green food from the normal diet of bran and oats resulted in the production of marked nephritis and certain other changes. The addition of a very small amount of green food to this diet kept the animals in permanent good health. This very marked effect of green food must be dependent on the vitamines contained in the greens. It is interesting to note that the chief organs primarily affected when the rabbit is deprived of certain vitamines are the kidneys. The early and intense albuminuria and the abundance of casts present in the urine of such animals give definite evidence of marked interference with renal function. This is later accompanied by a general wasting, and the condition, unless soon relieved, quickly terminates in death.

SUMMARY.

High protein feeding when accompanied by a little green food produces temporary changes in the metabolism of the rabbit, which are indicated by a marked rise in blood- and urine-urea. If this high protein diet is continued, these changes are no longer in evidence after about a month. The renal mechanism apparently adapts itself to the increased nitrogenous intake, so that examination of both blood and urine for nitrogenous waste products gives normal results.

A very high protein diet, when a little green food is also given, does not give rise to any changes of the nature of nephritis in the rabbit's kidney. The same diet in the absence of all green food very quickly produces marked albuminuria accompanied by the presence of abundant casts in the urine. This condition is very quickly relieved if a small amount of green food is given, but if green food is persistently withheld for some time after the nephritis is established, the animal dies. In no case could any evidence of chronic inflammatory changes be found on microscopical examination of the kidneys of such rabbits.

There is no evidence that high protein diet has any deleterious effect whatever on the kidneys of the rabbit; indeed, the kidneys very soon adapt

themselves to the new conditions. The absence of green food in the diet, however, very soon results in nephritis being produced, no matter what the protein content of the diet happens to be. These facts may have some bearing on the treatment of nephritis, for fresh vegetables may be more important than the usual abstention from protein food.

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ON THE COMBINED ACTION OF ANTIGENS AND ANTIBODIES ON THE BACTERICIDAL POWER OF DEFIBRINATED BLOOD.*

E. W. TODD, M.D., D.P.H.

From the Laboratories of the Institute of Pathology and Research, St. Mary's Hospital, London.

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THE bactericidal power of fresh defibrinated blood is frequently almost completely destroyed by the addition of a mixture containing an antigen and its homologous antibody. It can be shown by control experiments that the separate addition of antigen or antibody to the blood does not materially reduce its bactericidal action. The following experiments illustratrate this point.

Experiment 1.—Serum from a case of puerperal septicæmia due to hæmolytic streptococci supplied the antibody. Antigen was obtained by rapid centrifugalization of a 48 hours' broth culture of hæmolytic streptococci isolated by blood culture from the same patient. The bactericidal tests were carried out in slide-cells (Wright, Colebrook and Storer, 1923) with staphylococcus. The controls consisted of normal human serum and uninfected broth.

Four dilutions of a 16 hours' broth culture of staphylococcus were used in each test. The figures record the number of colonies which grew in 50 c.mm. of the mixtures after 24 hours' incubation in slide-cells.

* The expense of this research was partly defrayed by a grant from the Medical Research Council.

Approximate number of staphylococci planted in 50 c.mm. of the mixtures.	Number of colonies in 50 c.mm. of mixtures containing 8 parts of normal human blood and 1 part of each of the following fluids:			
	Normal serum + control broth.	Normal serum + centrifuged culture.	Normal serum + patient's serum.	Patient's serum + centrifuged culture.
752	80	55	57	+++
376	28	25	7	100
188	8	8	4	55
94	10	2	5	43

The mixture containing normal blood, patient's serum and antigen had a much smaller bactericidal action than any of the control mixtures.

Experiment 1*a*.—A similar result was obtained with the same patient's serum by a different technique. A culture of the streptococcus isolated from the patient was used as the test organism. The antigen was obtained by filtering a 4 days' broth culture of the same strain of streptococcus through a Berkefeld filter. Normal human serum and uninfected filtered broth were used as controls. Normal human blood was infected with suitable numbers of streptococci, and equal volumes of this infected blood were used to complete the mixtures. After 3 hours' incubation on a mixing machine equal volumes of the mixtures were plated out in blood agar and the number of colonies which developed after 24 hours' incubation were counted.

Number of colonies obtained from equal volumes of mixtures containing 20 parts of normal blood and 1 part of each of the following fluids:			
Normal serum + control broth.	Normal serum + filtrate.	Patient's serum + control broth.	Patient's serum + filtrate.
38	41	1	248

The destructive effect of antigen and antibody on the bactericidal power of the blood is apparent, and this occurred in spite of the fact that the patient's serum with control broth had a well-marked beneficial influence.

Experiment 2.—In the second experiment serum from a case of septicæmia due to *Staphylococcus albus* was used as antibody, and the antigen was obtained by centrifuging a 48-hour culture of the homologous staphylococcus at high speed. The clear supernatant broth was then heated at 60° C. for one hour. The tests were carried out in slide-cells and Ringer's solution was used to adjust the volumes.

Approximate number of staphylococci planted in 50 c.mm. of the mixtures.	Number of colonies in 50 c.mm. of mixtures containing 8 parts of normal human blood and 1 part of each of the following fluids:					
	Ringer's solution + normal serum.	Ringer's solution + patient's serum.	Ringer's solution + control broth.	Ringer's solution + centrifuged culture.	Normal serum + centrifuged culture.	Patient's serum + centrifuged culture.
840	71	66	+++	+++	+++	+++
420	55	37	89	88	105	+++
210	27	27	54	56	57	+++
105	20	14	19	47	40	98
27						

The mixture containing antigen and antibody again had a much lower bactericidal value than any of the control mixtures.

This experiment also demonstrates the toxic effect of broth on the bactericidal action of blood.

The toxic effect of a mixture containing an antigen and its homologous antibody on the bactericidal action of normal blood is probably a complex phenomenon in which many factors, including fixation of complement, are concerned. There is, however, one factor which is comparatively easy to investigate, namely the production of precipitate in the blood by the interaction of precipitin and precipitinogen. The following observations have been confined to this aspect of the phenomenon, and precipitates have been formed in the blood by three different methods:

(1) By the addition of precipitin and precipitinogen to normal human blood.

(2) By the addition of precipitinogen to blood which contains precipitin.

(3) In the third and most important method no extraneous fluids are added to the blood. The precipitin is contained in the blood used in the test, and the precipitinogen is supplied by the cocci which are used as the test organisms.

THE ADDITION OF PRECIPITIN AND PRECIPITINOGEN TO NORMAL HUMAN BLOOD.

The materials for the experiments which will be described under this heading were again obtained from cases of septicæmia. The experiments were similar to those already described, but they were carried a stage further, and the formation of precipitate in the antigen-antibody mixture was observed and the effect of its removal by centrifugalization was investigated.

Experiment 3.—The serum was obtained from a case of puerperal septicæmia infected with hæmolytic streptococci. The antigen was a 16 hours' broth culture of the homologous streptococcus, which was sterilized by heating for 1 hour at 60° C. A clear supernatant fluid was obtained from this dead culture by centrifuging. Equal parts of the patient's serum and antigen were incubated together at 37° C. for 48 hours, and the resulting precipitate was then removed by centrifuging. The action of this clear fluid was then compared with the action of freshly-mixed precipitin and antigen on the bactericidal power of normal blood in slide-cells.

Approximate number of staphylococci planted in 50 c.mm. of mixtures.	Number of colonies in 50 c.mm. of mixtures containing 8 parts of normal human blood and 1 part of each of the following fluids:			Number of colonies in 50 c.mm. of mixtures containing 8 parts of normal human blood and 2 parts of—	
	Ringer's solution + patient's serum.	Ringer's solution + centrifuged culture.	Patient's serum + centrifuged culture.	Ringer's solution.	Mixture of patient's serum and antigen after removal of precipitate.
272	18	120	+++	21	21
136	6	72	+++	12	8
68	7	26	+++	2	5
34	3	20	+++	0	3

The mixture containing precipitin and antigen was again of much lower bactericidal value than the controls, but the mixture containing precipitin and antigen which had been incubated and centrifuged to remove the precipitate gave a similar reading to the controls.

Experiment 4.—In this experiment a similar result was obtained by a different technique. Serum from a case of puerperal septicæmia due to hæmolytic streptococci was used as precipitin. A heterologous strain of hæmolytic streptococcus isolated from a different case of puerperal septicæmia was incubated at 37° C. for 5 days. The culture was then passed through a Berkefeld filter and the clear filtrate was used as antigen. A young broth culture of the streptococcus from which the filtrate was prepared was used as the test organism. Equal parts of the patients' serum and the filtrate were mixed together in a small glass tube and left on the bench at room temperature for 2½ hours. A good precipitate had then formed and 10 c.mm. of the mixture containing the precipitate was transferred to a glass capsule. The original tube was then centrifuged to remove the precipitate and 10 c.mm. of the resultant clear supernatant fluid was transferred to a second glass capsule. Three control capsules were prepared, the first containing 10 c.mm. of normal serum, the second 5 c.mm. of normal serum and 5 c.mm. of the patient's serum, and the third 5 c.mm. of normal serum and 5 c.mm. of the filtrate. Normal fresh defibrinated blood was then infected with a convenient number of hæmolytic streptococci and 100 c.mm. of this infected blood was added to each of the five capsules, which were immediately sealed and incubated on a mixing machine. After 3 hours' incubation 50 c.mm. of fluid from each of the capsules was plated out in blood agar and the number of colonies which had developed after 24 hours' incubation was counted.

Approximate number of streptococci planted in 50 c.mm. of blood.	Number of colonies from 50 c.mm. of mixtures containing 20 parts of normal human blood and 1 part of each of the following fluids:		Number of colonies from 50 c.mm. of mixtures containing 20 parts of normal human blood and 2 parts of—		
	Normal serum + patient's serum.	Normal serum + filtrate.	Normal serum.	Patient's serum and filtrate *uncentrifuged.*	Patient's serum and filtrate *centrifuged.*
210	1	26	1	125	1

It appears from these experiments that the bactericidal power of normal blood is greatly reduced by the presence of precipitate in the hæmo-bactericidal test. It is probable that this toxic effect is due to the presence of finely divided particles in the blood, since a similar result can be obtained if normal serum containing finely divided coagulated particles is added to normal blood.

Experiment 5.—Normal human serum was passed rapidly through a very hot capillary glass tube so that small finely divided particles of coagulated protein were formed in the serum. The appearance of serum treated in this way is similar to that of precipitates formed by the interaction of precipitin and precipitinogen. The action of the partially coagulated serum on the bactericidal power of normal blood was tested in slide-cells with staphylococcus.

Approximate number of staphylococci planted in 50 c.mm. of the mixtures.	Number of colonies in 50 c.mm. of undiluted normal blood.	Number of colonies in 50 c.mm. of mixtures containing 4 parts of normal human blood and 1 part of—	
		Unheated normal serum.	Partially coagulated normal serum.
408	37	35	150
204	16	17	98
102	5	5	43
51	4	5	16

The blood which contained finely divided particles of coagulated serum had a lower bactericidal value than either of the controls. It is therefore probable that the toxic action of homologous antigens and antibodies on the bactericidal action of normal blood is largely due to the deposit of precipitate in the blood during the bactericidal test.

THE EFFECT OF ADDING ANTIGEN TO BLOOD WHICH CONTAINS THE HOMOLOGOUS ANTIBODY.

In the experiments which will be described under this heading, the action of an antigen on the bactericidal power of normal blood was compared with the action of the same antigen on blood which contained the homologous antibody.

Experiment 6.—The patient had received a subcutaneous injection of horse-serum a few weeks before the test. The action of normal horse-serum on the bactericidal power of the patient's blood was tested in slide-cells with staphylococcus. A control experiment with normal blood was put up at the same time.

Number of colonies in 50 c.mm. of mixtures containing 4 parts of *patient's blood* and 1 part of—		Number of colonies in 50 c.mm. of mixtures containing 4 parts of *normal blood* and 1 part of—	
Ringer's solution.	Normal horse-serum.	Ringer's solution.	Normal horse-serum.
52	+++	51	60
41	+++	27	37
22	++	19	23
17	100	14	24

The addition of normal horse-serum to the patient's blood caused a considerable fall in bactericidal power. On the other hand, the addition of normal horse-serum to normal blood did not cause any material alteration in bactericidal power.

Similar results were obtained by causing the formation of precipitate in syphilitic blood by the addition of heart extract.

Experiment 7.—Defibrinated blood was obtained from a patient whose serum gave strongly positive Wassermann and Sygma reactions. One-tenth of a c.c. of alcoholic extract of human heart was evaporated to dryness and the deposit was emulsified in 4·5 c.c. of normal saline. The action of this emulsion on the bactericidal power of the patient's blood was tested in slide-cells with staphylococcus. A control experiment with normal blood was put up at the same time.

Number of colonies in 50 c.mm. of mixtures containing 4 parts of *patient's* blood and 1 part of—		Number of colonies in 50 c.mm. of mixtures containing 4 parts of normal blood and 1 part of—	
Normal saline.	Heart extract.	Normal saline.	Heart extract.
77	+++	64	60
44	+++	40	31
14	50	11	10
9	40	7	11

The formation of precipitate in the blood was accompanied by a marked fall in bactericidal power.

THE FORMATION OF PRECIPITATES IN HÆMO-BACTERICIDAL TESTS WITHOUT THE ADDITION OF ANY EXTRANEOUS FLUIDS.

A broth culture of *Staphylococcus aureus* was incubated for 10 days at 37° C. The culture was then killed by heating for 1 hour at 60° C., and after rapid centrifuging a clear supernatant fluid was obtained. A rabbit was inoculated with this antigen intravenously. After three inoculations of 0·5 c.c., 1 c.c. and 1·5 c.c. the rabbit's serum produced a precipitate with the antigen up to a dilution of 1 in 24 of the serum.

The serum of a normal rabbit which contained no precipitin was used as a control. These two sera were infected with suitable dilutions of a young broth culture of the strain of staphylococcus used in the preparation of the antigen. The infected sera were then filled into slide-cells in exactly the same way as has been described for the hæmo-bactericidal test. The slide-cells were examined after 16 hours' incubation. Colonies of staphylococci had grown in all the dilutions in both sera, but there was a marked difference in appearance. The colonies growing in the normal serum had clearly defined edges and were surrounded by clear serum which was not changed in its appearance by the growth of the colonies. In the serum of the inoculated rabbit similar colonies with clearly defined edges had grown out, and these colonies were surrounded by a narrow zone of clear serum. Outside this clear zone each colony was surrounded by a hazy white halo, which radiated from the colony to a distance of three or four times the diameter of the colony, and gradually faded away into the surrounding clear serum without any clearly defined outer margin. After 24 hours' incubation the haloes had increased in density and in circumference, but the zone of clear serum immediately surrounding the colony was maintained. Microscopical examination of the haloes showed that they were not due to spreading bacterial growth, but were composed of a granular substance which exactly corresponded in its microscopical appearance with the precipitate obtained by mixing together the inoculated rabbit's serum and clear sterile filtrate from a broth culture of staphylococcus. By careful examination similar indistinct haloes can be seen round colonies of staphylococci growing in *blood* which contains precipitin. The appearance is, however, obscured by the red blood-corpuscles.

Dudgeon and Bamforth (1924–25) have shown that the serum of many apparently normal rabbits contains precipitin for *Staphylococcus aureus* antigens. This is an important fact in connection with these observations, as it is necessary

to choose a rabbit whose serum is free from precipitin as a control. In the experiment described above the control serum contained no precipitin, and staphylococcal colonies growing in this serum were not surrounded by haloes. The serum of the rabbit used for inoculation contained some precipitin before inoculations were commenced. The bactericidal power of the blood of these two rabbits was compared, and observations were made on the precipitin content of the sera at the same time.

Bactericidal Power and Precipitin Content of Rabbits' Blood BEFORE *Inoculation.*

The precipitin content of the sera was determined by mixing constant volumes of antigen with falling dilutions of serum.

Dilution of serum :—	1 in 2.	1 in 4.	1 in 8.	Serum control.
Serum before inoculation	+	+	0	0
Control serum	0	0	0	0

The bactericidal power of the two bloods was tested with staphylococcus in slide-cells. The figures given below are averages of triplicate readings:

Approximate number of staphylococci planted in 50 c.mm. of blood.	Number of colonies in 50 c.mm. of—	
	Rabbit's blood before inoculation.	Control rabbit's blood.
56	28	17
28	16	8
14	8	3
7	3	1
Average percentage killing	45·9	73·0

The blood which contained no precipitin was more bactericidal than the blood which contained precipitin.

Bactericidal Power and Precipitin Content of Rabbits' Blood AFTER *Inoculation.*

Dilution of serum :—	1 in 3.	1 in 6.	1 in 12.	1 in 24.	1 in 48.	Serum control.
Inoculated rabbit's serum	++	+	tr.	tr.	0	0
Control rabbit's serum	0	0	0	0	0	0

Approximate number of staphylococci planted in 50 c.mm. of blood.	Number of colonies in 50 c.mm. of—	
	Inoculated rabbit's blood.	Control rabbit's blood.
144	98	16
72	57	9
36	27	4
18	14	3
Average percentage killing	27·4	88·1

This experiment again shows the correlation between the precipitin content and the bactericidal power of the blood.

A similar result was obtained with human blood. Dudgeon and Bamforth have shown that the serum of patients suffering from serum sickness frequently gives a positive precipitin reaction with *Staphylococcus aureus* filtered antigen. Their control experiments with filtered antigens prepared from *B. typhosus*, *B. paratyphosus B*, *B. coli* and *B. proteus* gave negative results. The blood of a patient who had suffered from serum sickness a few weeks previously was therefore obtained, and his serum was found to give a positive precipitin reaction with *Staphylococcus aureus* filtered antigen. The bactericidal power and precipitin content of this patient's blood and of normal blood were compared.

Precipitin Test.

Dilution of serum :—	1 in 3.	1 in 6.	1 in 12.	Serum control.
Patient's serum	++	++	+	0
Control serum	0	0	0	0

Bactericidal Test.

Approximate number of staphylococci planted in 50 c.mm. of blood.	Number of colonies in 50 c.mm. of—	
	Patient's blood.	Control blood.
1216	110	4
608	72	0
304	56	0
152	16	0

The bactericidal power of the blood containing precipitin was lower than that of the normal control. This patient was under observation for several weeks. During this time the bactericidal power of his blood was tested frequently with staphylococcus, and it was consistently low in comparison with the blood of a number of normal individuals. A few tests were also done with *Streptococcus pyogenes* and with non-hæmolytic streptococci. With these organisms the patient's blood was not obviously subnormal in bactericidal action as it was with staphylococcus.

These experiments show that precipitates can be formed in hæmobactericidal tests without the addition of any extraneous fluids, the precipitin being supplied by the blood of an inoculated patient and the precipitinogen being supplied by the staphylococci growing in the blood. It has already been shown that the presence of precipitates in hæmobactericidal tests is destructive to the bactericidal action of the blood. It therefore seems probable that the bactericidal power of blood from an inoculated patient will be reduced by the formation of precipitate in the hæmobactericidal test if the inoculation has produced precipitin in the blood.

ATTEMPT TO DEMONSTRATE AN INCREASE IN THE BACTERICIDAL POWER OF RABBITS' BLOOD FOLLOWING VACCINATION.

Unfortunately the bactericidal power of rabbits' blood is subject to considerable variation, and it is therefore necessary to make a large number of observations to obtain reliable results. In the following experiment two normal rabbits whose sera gave negative precipitin reactions with staphylococcal filtered antigen were selected. The bactericidal power of their blood for staphylococcus was tested in slide-cells at intervals of 3 days for a period of 18 days. The percentages of implanted staphylococci killed by the two bloods are recorded below. Each figure is calculated from the average of 3 separate observations, 4 dilutions being used in each observation, so that the figures represent the average of 12 readings. The serum of the two rabbits was tested for precipitin at the beginning and at the end of this series of observations with negative results.

Percentage of staphylococci killed by control rabbit's blood.	Percentage of staphylococci killed by rabbit's blood *before* inoculation.
68·0	72·0
62·1	83·7
73·0	76·8
66·9	89·2
30·1	65·0
74·8	65·1

There was considerable variation in the bactericidal action of both the rabbits' blood from day to day, but it is noteworthy that five times out of six the control rabbit's blood had the lower bactericidal power.

Three intravenous injections of 1000 million, 2000 million and 3000 million of staphylococcal vaccine were given to one of the rabbits at 10-day intervals. Three days after the last injection a second series of observations on the bactericidal power of the rabbits' blood was commenced. This series of observations extended over 20 days and readings were taken at intervals of 3 days.

Percentage of staphylococci killed by control rabbit's blood.	Percentage of staphylococci killed by inoculated rabbit's blood.
62·3	9·4
78·3	56·6
50·8	16·1
76·8	73·4
88·1	51·1
45·1	39·2
40·7	63·5

There was no improvement in the bactericidal power of the inoculated rabbit's blood, although the agglutination titre of its serum was 1 in 1600 with emulsions of staphylococcus. In this series of observations the relative values of the bloods were reversed, as six times out of seven the control rabbit's blood had the greater bactericidal power, so that if any change can be credited to the vaccination, it appears to be one of lowered bactericidal power. The serum of the two rabbits was tested for precipitin as before, and staphylococcus filtered

antigen was found to give a positive precipitin reaction with the inoculated rabbit's serum up to a dilution of 1 in 12. The precipitin reaction with the control rabbit's serum was negative. Haloes of precipitate were formed round colonies of staphylococci growing in the serum of the inoculated rabbit, while this phenomenon was not observed in the control serum.

It is probable that the bactericidal power of the inoculated rabbit's blood was reduced by the formation of precipitate in the hæmo-bactericidal test.

SUMMARY.

The formation of precipitates in hæmo-bactericidal tests is destructive to the bactericidal power of the blood.

When the bactericidal power of blood which contains precipitin is tested on the homologous organism, precipitinogen is liberated by the growth of the organism in the blood, and the resultant precipitate reduces the bactericidal action of the blood.

Thus when the bactericidal power of a patient's blood is tested against staphylococci a low bactericidal value may be expected if, owing to past infection, inoculation, or any other cause, the blood contains staphylococcal precipitin.

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THE ACTIVATION OF FILTRATES OF THE ROUS SARCOMA BY AN AGENT IN BROTH CULTURES OF JENSEN'S RAT SARCOMA.

S. L. BAKER.

From the Bland-Sutton Institute of Pathology, Middlesex Hospital, London.

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SINCE the original paper of Gye (1925) several other workers have repeated his experiments, with variable results. Two very complete accounts concern experiments on the first part of the problem—that is, on the demonstration of two factors in the Rous sarcoma. In the first of these, Harkins and his co-workers (1926) failed to confirm Gye's results because they could not with any certainty inactivate the tumour filtrates with chloroform—even when used in excessive amounts. Two other workers, Mackenzie and Illingworth (1926), have confirmed Gye's experimental results with primary "cultures" of the

Rous tumour, but have been unable to obtain any activation of the chloroformed filtrate by sub-cultures of the tumour. They had no difficulty in inactivating the filtrate by chloroform.

The present communication deals with the second part of the problem—that is, the demonstration of an activating factor (" virus ") in the fluid from " cultures " of mammalian tumours. In this work the activation of chloroformed filtrates of the Rous fowl sarcoma by fluid from anaerobic " cultures " of Jensen's rat sarcoma (J.R.S.) has been studied. These " substitution " experiments present a somewhat clearer issue than experiments with cultures of the Rous tumour, since there is no possibility of the piece of mammalian tumour by itself producing a neoplasm in the fowl, while there must always be some uncertainty as to the length of time that the fowl tumour may retain its activity in the culture medium.

TECHNIQUE.

The technique of Gye (1925, 1926) has been followed throughout. Water-clear filtrates, prepared by filtering the ground tumour (2·5 gm. per 100 c.c. of saline) through paper-pulp and sand, have been used. Primary cultures of the rat sarcoma have been made in Hartley's broth, to which has been added KCl and rabbit serum; for subcultures chick embryo has been added to the medium.

Inactivation of the filtrate has been carried out by adding sufficient chloroform to ensure saturation, and incubating at 37°C. in a stoppered tube with occasional agitation for 3–4 hours.

Throughout this series the inoculation of the experimental and the control dose has been made into the pectoral muscles on opposite sides of the same bird. This appeared more reliable than the use of separate birds, since it eliminates individual variations in susceptibility. The keel-bone of the fowl forms a practically impassable barrier to the spread of the tumour from one side to the other. A very large tumour may spread across the mid-line in the subclavicular region where the bony partition is absent, but this form of spread is always obvious, and no confusion has arisen from bilateral inoculation of the birds.

The chief technical difficulty that has arisen has been the inactivation of the filtrate by chloroform. In this respect the different results obtained by the two groups of workers mentioned above is peculiar, and obviously indicates some very significant, but, at present, uncontrolled factor in the process. My own experience in this matter falls between that of these two workers. I have succeeded in inactivating the filtrate on many occasions, but have been much troubled by the erratic action of the chloroform. Numerous experiments have been vitiated by the persistent activity of the filtrate after 3 hours' incubation with chloroform, and on one occasion it was quite active after 4 hours' incubation.

I do not think that the failure to inactivate is due to the excessive purity of the chloroform as suggested in Gye's paper (1926), since I have been using ordinary stock chloroform containing traces of chlorine, hydrochloric acid and doubtless other impurities.

RESULTS.

The results obtained show activation of the chloroformed filtrate by several primary cultures of the rat tumour and in two cases by subcultures. This activation has consisted for the most part in a striking quantitative effect, since on dissection the control side has usually shown a minute tumour nodule, even when apparently negative externally.

A, *B* and *C* (Fig. 1) show effects with broth from primary cultures of J.R.S. The birds were inoculated as follows:

A, right breast 0·5 c.c. of chloroformed filtrate. Left breast 0·5 c.c. of chloroformed filtrate + 0·5 c.c. of supernatant broth from a tube in which a piece of J.R.S. had been incubated anaerobically for 3 days.

B and *C* received into right breast 0·5 c.c. of chloroformed filtrate + 0·5 c.c. of broth in which a piece of J.R.S. had been incubated for 5 days. Into left breast 0·5 c.c. of chloroformed filtrate + 0·5 c.c. of broth from an uninoculated tube incubated 5 days.

Experiment 175/25 (Fig. 1) shows an experiment in which a second subculture of J.R.S. was used; this was made by subculturing a few drops of supernatant broth from a primary culture of J.R.S. prepared as indicated in the previous experiment; this subculture was incubated for 5 days and a few drops again subcultured and incubated for 6 days.

The birds *D* and *E* were inoculated as follows:

Right breast chloroformed filtrate 0·5 c.c. + J.R.S. second subculture 0·5 c.c.

Left breast chloroformed filtrate 1 c.c.

The bird *F* received into the left breast chloroformed filtrate 0·5 c.c. + broth from an uninoculated tube containing chick embryo 0·5 c.c. The bird *G* received the same injection as *F* into the left breast, and into the right breast 0·5 c.c. of untreated filtrate. This experiment shows that an activating agent is present in the broth-tube of the second subculture of a piece of J.R.S., that the uninoculated broth containing chick embryo contains no activating agent, and that chicken *G* is susceptible to the untreated filtrate.

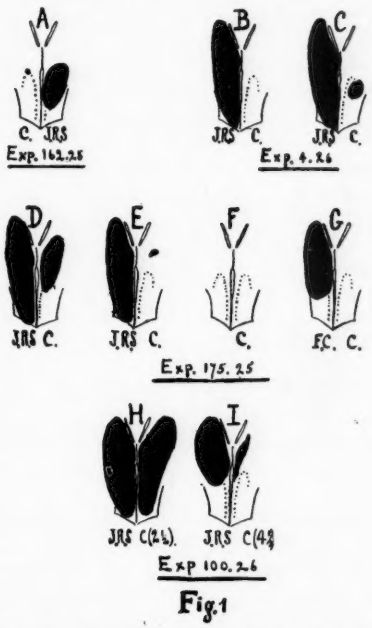

FIG. 1.—Experiments showing the activating effect of fluid from broth cultures containing pieces of Jensen's rat sarcoma upon inactivated filtrates of the Rous fowl sarcoma. J.R.S. = The side treated with fluid from the cultures of Jensen's sarcoma + chloroformed filtrate of the Rous sarcoma. C = The control side (*vide* text for nature of control) in each case. C (2½) = Filtrate chloroformed for 2½ hours and still very active. C (4½) = The same filtrate chloroformed for 4½ hours. FC. = Untreated filtrate.

This experiment is the best evidence that I have obtained of transmission

of a virus through cultures, but it cannot be said that it is very conclusive, since the dilution of the original broth would not amount to more than 1/1000. In many other experiments with more remote subcultures I have failed to demonstrate any activation. Murphy (1926) reports that he obtains an activating agent from cultures of chick-embryo; I have never found this in the chick-embryo medium I have been using.

Experiment 100/26 shows the only other evidence I have been able to obtain of transmission of a virus through cultures; it also illustrates the difficulty I have had in inactivating the filtrates with chloroform.

The same filtrate was used in the inoculation of both the birds in this experiment, but in the case of the bird *H* it was only incubated with chloroform for 2½ hours, while in the case of bird *I* it was chloroformed for 4½ hours.

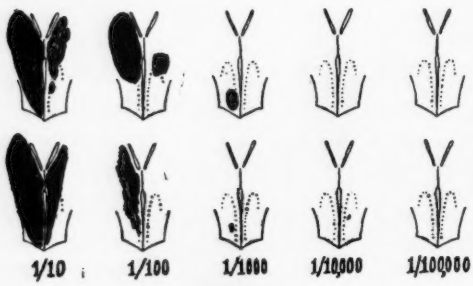

Fig. 2.

Fig. 2.—Experiment showing that fluid from the cultures of Jensen's rat sarcoma can activate filtrate attenuated by dilution alone. The filtrate was diluted 1/10–1/100,000, as indicated in diagram. A pair of birds was used for each dilution. Each bird received into the right breast 0·5 c.c. of the diluted filtrate + 0·25 c.c. of the supernatant fluid from a "primary culture" of Jensen's sarcoma, and into the left breast 0·5 c.c. of the diluted filtrate alone.

The first subculture of a J.R.S. was used in this case; this was prepared by inoculating one drop of supernatant fluid from a primary culture into a tube of chick-embryo broth—dilution of primary culture about 1/400.

The bird *H* was inoculated into the right breast with filtrate chloroformed 2½ hours 0·5 c.c. + J.R.S. first subculture 0·5 c.c. Into the left breast was inoculated the same chloroformed filtrate 0·5 c.c. + uninoculated chick-embryo broth 0·5 c.c. The bird *I* was inoculated in the same manner, but filtrate which had been chloroformed 4½ hours was employed. These results show that the filtrate chloroformed for 2½ hours was very active, and that even after 4½ hours' chloroforming it was only partially inactivated. There appears to be a definite stimulation of the partially inactivated filtrate by broth from the subculture of J.R.S.

The difficulty of obtaining consistently negative controls can be seen from the above experiments, as frequently where one control bird is completely negative a companion may show a small tumour. This has been my experience with the chloroform inactivation throughout, and gives the impression of a very small margin of safety between successful inactivation and complete destruction of the filtrate. I have no doubt that with different chloroform or by the adjustment of some unknown factor in the technique a series of consistently negative controls could be obtained, but even with such controls there would still appear to be some doubt as to the complete extermination of the Rous infecting agent.

A question which has been considered by other workers, and which naturally arises, concerns the differential action of the chloroform. Do the experimental results necessitate the assumption of a relatively greater destruction of "virus" than of "specific factor," or may the chloroform merely act by producing a general attenuation of the Rous tumour filtrate?

The following experiment was planned to discover whether a culture of J.R.S. would activate filtrate attenuated by simple dilution which must act equally on all the constituents.

Rous tumour filtrate was progressively diluted 1/10, 1/100, 1/1000, 1/10,000, 1/100,000. Into the left pectoral muscles of two birds was injected 0·5 c.c. of a 1/10 dilution, while into the right pectorals of same was injected 0·5 c.c. of this dilution + 0·25 c.c. of supernatant fluid from a primary culture of J.R.S. The same process was carried out with the other dilutions, a pair of birds being used in each case. It is seen from Fig. 2 that there is a larger tumour on the right side (+ J.R.S.) in all cases, and that the end-point for growth on the left side is about 1/100, and on the right side about 1/1000.

It appears, therefore, that fluid from primary cultures of J.R.S. can activate filtrate attenuated by dilution.

DISCUSSION.

The general behaviour of the Rous tumour—its propagation by dilute cell-free filtrates, the persistence of infectivity for long periods in dried material and the destruction of the infectivity by various bactericidal agents—leave little doubt in the minds of many of those who have worked with it that a living virus is concerned in its production.

In the production of the tumours in the substitution experiments of Gye and in those considered above there are obviously two factors involved, one in the culture and one in the chloroformed filtrate. Assuming that a virus is involved in the production of the Rous tumour, as most workers believe, there appear to be two possible explanations of the experimental results: either—

(*a*) The culture of mammalian tumour provides a virus and the chloroformed filtrate of the Rous tumour an activating factor; or—

(*b*) The chloroformed filtrate of the Rous tumour contains an attenuated virus and the culture of mammalian tumour provides activating factor.

In favour of (*a*) are—

(i) Gye's demonstration of the propagation of the activating agent in the cultures through numerous subcultures.

(ii) The direct observation of certain bodies by Barnard.

In favour of (*b*) are the facts that—

(i) One cannot exclude the possibility of attenuated virus in the chloroformed filtrates.

(ii) A primary "culture" of J.R.S. will stimulate a filtrate attenuated by dilution alone.

(iii) Chemical substances which will stimulate tumour-formation have been demonstrated in tumour and tissue extracts (Chambers and Scott, 1926; Flu, 1926; Murphy, 1926).

Whatever may be the final interpretation of these substitution experiments, the hypothesis that malignant neoplasms in general are the result of the action of a living virus has obviously become much more tenable since the demonstration of a mechanism involving the combined action of two factors in the particular case of the Rous sarcoma.

SUMMARY.

(1) It is shown that an agent is present in cultures of Jensen's rat sarcoma which will activate chloroformed filtrates of the Rous chicken sarcoma No. 1.

(2) In two cases this agent has been demonstrated in a first and a second subculture.

(3) Further, an agent in primary cultures of the rat sarcoma will activate filtrate attenuated by dilution.

(4) No definite conclusions are reached as to the nature of the activating agent in the cultures.

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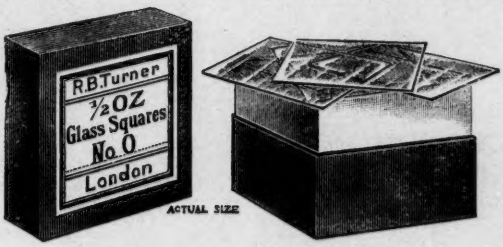

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